Locomotor sensitization to amphetamine is encoded by nucleus accumbens Fos-expressing neuronal ensembles in a context-specific manner

by

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Abstract

Learned associations between drug rewards and complex environmental stimuli are encoded in the brain and guide behavior. Studies analyzing activated neuronal ensembles in animal models of addiction-related behaviors, such as locomotor sensitization, have revealed unique molecular and cellular alterations that may encode learning. Here, we examined activated neurons expressing the activity marker Fos in the nucleus accumbens after locomotor sensitization, and aimed to (I) characterize subtypes of neurons recruited to encode learned associations, and (II) determine if Fos-expressing neurons encode learned associations that mediate context-specific locomotor sensitization. Rats were repeatedly administered amphetamine (2 mg/kg, i.p.) or saline (1 ml/kg i.p.) across 5-days, and after 1-week abstinence were acutely injected with amphetamine (0 or 1 mg/kg, i.p.) prior to a locomotor activity test. We examined co-expression of Fos with other markers to characterize the Fos-expressing neuronal ensemble found in rats exhibiting locomotor sensitization. Examining *Fos*-expressing neuronal ensembles for dopamine-receptor distribution, we found increased recruitment of *D1-receptor*-expressing neurons. Examining co-expression of the Fos with ΔFosB, a protein marker we showed labels neurons previously activated during repeated administration, we found increased recruitment of ΔFosB-expressing neurons to the Fos-expressing neuronal ensemble. Next, we examined whether locomotor sensitization was context-specific by repeatedly administering amphetamine in either the testing context or a distinct context. Sensitized amphetamine-induced locomotor activity, Fos expression, and Fos/ΔFosB co-expression were induced only when rats were

conditioned and tested in the same context. We further established that Fos-expressing neurons played a causal role in context-specific locomotor sensitization by ablating activated neurons with the Daun02 inactivation procedure. Microinjection of Daun02 into the accumbens shell attenuated sensitized locomotor activity when rats were conditioned in the same context in which they were tested, while rats conditioned in a different context from which they were tested had no change in locomotor activity, despite confirmed ablation of the previously activated neurons. These data highlight a role of activated neuronal ensembles in encoding important learned associations between context and drug and suggest future targets for addiction-related learned behaviors.

Primary readers: Bruce Hope, Haiqing Zhao

Secondary readers: Patricia Janak, Scott Russo

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Preface

1. A note about the process

Over the years, my expectations about what it is to have a PhD have changed drastically from the veiled, romantic imagery instilled in me at a young age. Though still enamored by the prestige and immortality a PhD and published research offers, I ultimately chose to get a PhD with the ambitious hope of answering some of the many questions posed within me about human motivation and behavior. Instead, I primarily learned of the literal blood, sweat, and tears that go into such a large composition, stealing some of the romance, but reinforcing the respect I have for those who live at the edge of knowledge. I have learned much about myself, the world, biology, and the brain, and I cherish the knowledge and skills I have accumulated the last 6-years. I choose to have no regrets (see Figure A, (Eatock, 2013)).

Figure A.

2. Personal acknowledgements

I could not have completed this work without the support and sacrifice of many. I have been blessed to accumulate a fantastic, dynamic, extensive network of family,

friends, and colleagues who have tirelessly supported my lifelong indulgence in education.

Most personally, I would like to thank my family. My sisters have provided me with invaluable life lessons found in both our striking similarities and notable differences. They motivate me to push myself, acting as cheerleader and teammate. I am blessed with wonderful parents who continuously enable my dreams, no matter how absurd or fleeting. In all that I do, I feel the resounding pride and support of my father and the unwavering care and strength of my mother. The love and patience of my immediate and extended family throughout this dissertation work was exemplary. I have also been blessed to have cared for two amazing cats during my PhD, who helped make me feel purposeful, and complete, and grounded. With my family's unwavering support, I have been gifted permission to take fearless leaps into the unknown.

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to learn, lead, and collaborate with dozens of incredible people. Their contributions to my research and career opportunities have shaped me in a permanent way.

Finally, I must emphasize that the greatest contribution for the advancements in my discoveries were from the animal subjects that provided the ultimate sacrifice for my research and animal research before me. As a partial redress, I dedicate my thesis to research animals, in particular rat #308. Throughout my scientific career, working with animals has forced me to be most thoughtful about my experimental design, made parts of work a pleasure, and poignantly taught me about humanity. I am forever appreciative.

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It would have been impossible to start this work without the solid scientific foundation laid out by researchers before me, the resources and facilities provided by the NIH, and the opportunities to train and collaborate with other labs before and while executing my PhD. In particular, I thank the previous members of the Hope Lab for paving the way for my research by developing technologies and uncovering interesting questions with their discoveries. I thank NIDA for its top-tier resources, through which I was able to outsource reliable animal husbandry, transgenic rat production, and administrative duties that were essential to the success of my experiments. Additionally, I am appreciative of the generosity of neighboring labs for the occasional

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Introduction

1. Learned associations and addiction

Associations made between environmental stimuli and reward shape behavior through conditioned learning (DeWitt et al., 2015; Torregrossa and Taylor, 2016). This type of learning is used to meet basic needs of survival, for example, returning to locations with known food sources (Nairne et al., 2011). However, systems for learned associations can be used to reinforce mal-adaptive behavior. In the case of human addiction, drug-seeking and drug-taking behavior can override pro-adaptive behaviors, such as complying with social norms (Cadet et al., 2014). In many instances, the desire to quit these behaviors is not enough to stop the motivation to take the drug.

Drug cravings continues even after rewarding effects of the drug are no longer considered euphoric (Russel, 1976; Baker et al., 1987), and are often triggered by drug-associated stimuli (Miller, 2013a). Even re-exposure to subconsciously drug-associated stimuli can drive relapse behavior (Childress et al., 2008), often after long periods of abstinence (Miller, 2013a). For many cases of addiction, mal-adaptive behaviors and relapse take a toll on the emotional and economic prosperity of addicts, their families, as well as society (Godfrey et al., 2004; NIDA-InSite, 2017). Research to understand and manipulate the biological underpinnings of learned associations in addiction will help alleviate these burdens, but additionally have the potential to benefit other diseases involving memory by uncovering basic mechanisms that encode learning.

2. Model organisms to study the brain

To ask questions about mal-adaptive learned associations driving addiction, studies use model organisms from a range of phylogenetic relatedness to humans depending on the questions they aim to answer about addiction (Miller, 2013b). Lower order organisms

(flies, worms, fish) can be useful for exploring genetic influences on learned associations by manipulating gene expression easily, but with severe limitations in comparing central nervous system adaptations (Teach.Genetics, 2013). Higher order organisms, such as primates, offer parallel cognitive and learning abilities to humans and offer almost analogous brain anatomy. However, primates mature slowly with long life expectancies making it difficult to perform genetic or developmental manipulations and often making studies cost prohibitive. For these reasons, rodents are more commonly used for modeling addiction behavior and studying relevant human brain changes (Miller, 2013b).

Rodents are genetically similar to humans offering reasonably equivalent brain systems that encode similar conditioned behaviors. Analogous proteins, cell types, brain structures, and behavior expression are seen for many diseases including addiction. Most common rodent models include mice and rats, which each have slight advantages and disadvantages (Teach.Genetics, 2013). Mice have a longer history of genetic manipulation making answers to developmental and cellular mechanism questions more available, while rats tend to have more easily discerned cognitive abilities. However, extreme similarities between rats and mice allow for many generalizations between rodent models, offering a rich literature for studying learning. In the following we will not explicitly indicate findings for rats or mice unless they are found to be different, in which case findings for rats are used.

3. Behavior models of addiction

Rats have been shown consistently to model two major types of learning used in addiction studies: operant learning and Pavlovian learning (Garcia Pardo et al., 2017).

Both types of learning require learned associations to form in the brain between stimuli and reward, but encode different approaches (Bouton, 2007).

Operant learning is goal-directed learning, where rats perform voluntary responses to obtain rewards or avoid punishments. In many operant learning experiments studying learned associations, rats are taught to self-administer rewards by interacting directly with cues, for example pressing a lever to receive food pellets. A variety of observations and manipulations of this learned self-administration during acquisition, abstinence, extinction, and reinstatement after extinction, as well as tests on other appetitive and aversive operant learning tasks, have uncovered lots about encoding of goal-directed learning.

Contrasting goal-directed learning, Pavlovian learning is an involuntary, conditioned learning that allows for neutral stimuli to become associated with inherently rewarding properties of drugs upon experimenter-administration (Bouton, 2007). One example of traditional Pavlovian addiction-related behavior includes conditioned-place preference. In conditioned-place preference experiments, a novel environment is paired with drug administration, and conditioned learning leads to observable preferences for the reward-paired context. Factors such as dose, duration, frequency and context all play a role in how quickly conditioned learning occurs (Bouton, 2007; Schuweiler et al., 2018b). Observation and manipulation of this learned preference during acquisition, extinction, and reinstatement have identified relevant brain areas, cell types, and neuronal ensembles related to learned associations in rodents. A second, important type of Pavlovian conditioned learning is behavioral sensitization.

3.1. Behavioral sensitization to stimulants

Behavioral sensitization occurs when intermittent, repeated administration of a stimulus results in progressive amplification of a behavioral response (Robinson and Becker, 1986). After conditioned learning has occurred to establish behavioral sensitization, for example after repeated amphetamine administration in a novel context, subsequent, acute injections of amphetamine should elicit behavior normally seen at higher doses of the drug (Neill et al., 1974; Kalivas et al., 1993). It is important to avoid neurotoxicity from extreme drug exposure to study behavioral sensitization (Robinson and Becker, 1986; Yoon et al., 2016). Different behaviors can be sensitized preferentially at different rates by different sensitization protocols explained more below.

It is hypothesized that the phenomenon of behavioral sensitization is a product of neural adaptations of the brain's reward circuitry similar to adaptations occurring in addiction learning (Robinson and Becker, 1986; Kalivas et al., 1993; Schuweiler et al., 2018a). Because repeated administration often occurs with a variety of novel stimuli, such as injections, handling, or environment used to record behaviors, for many studies it can be difficult to dissociate learned aspects of behavioral sensitization from pharmacokinentic aspects (Robinson and Becker, 1986).

Fortunately, there are ranges of behaviors that become sensitized with repeated administration of stimulants that seem to be expressed by different aspects of learning and drug effects. The administration method, dose, duration, administration frequency, withdrawal period, test conditions, and contextual stimuli all play a role in the behavioral sensitization response. Intermittent, repeated amphetamine administration is known to induce sensitization of restrictive or hyperactive behaviors. Stereotyped head-

bobs, licking behaviors, and excessive grooming are varieties of restrictive sensitization behavior (Neill et al., 1974), while locomotor sensitization, most often measured as distance traveled, relies on sensitization of hyperlocomotion. Locomotor sensitization is proposed to later promote the seeking and self-administration of psychostimulants, making it an addiction-related behavior that may underlie the transition from casual drug use to compulsive drug taking and abuse (Vezina et al., 2002). Additional measures of locomotor sensitization can be made by quantifying number of rears, rotations (Robinson and Becker, 1986). More recently even ultrasonic vocalizations of rats have been used to determine sensitization (Costa et al., 2015; Kaniuga et al., 2016). Different sensitization protocols have tendencies to elicit different sensitized behavior outputs, with higher doses during repeated administration or testing typically inducing more restrictive stereotypic effects and lower doses during repeated administration and testing inducing hyperlocomotor effects, and a scoring system for stereotypic activity quantifies these differences (Ellinwood and Balster, 1974). It is important to note that sensitized restrictive stereotypies can interfere with locomotor sensitization, and precautions to address the interference may be required to properly measure sensitization.

To test drug doses that induce locomotor sensitization, comparisons between rats repeatedly injected with a simulant are made to rats repeatedly injected under the same conditions with saline to control for drug-specific effects versus non-drug protocol effects or acute stimulant effects. Still, there is no single protocol for sensitization, and many variations elicit robust locomotor sensitization (Badiani et al., 2000). However, it is well documented that with the right repeated administration protocol and controls,

increased locomotor activity to amphetamine-induced sensitization can be context-specific (Badiani et al., 1997).

3.2. Context-specific locomotor sensitization

When a novel environment is paired with a stimulant, such as amphetamine, properties of the context have been known to induce a conditioned locomotion, presented as locomotor sensitization with no drug on board. In these cases, associated stimuli from the context are enough to induce a sensitized locomotor response. However, presence of conditioned locomotion is not a requirement of context-specific locomotor sensitization (Robinson and Becker, 1986; Badiani et al., 1995; Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002).

In studies controlling for learned associations that induce locomotor sensitization in a context-specific manner, rats can be repeatedly administered amphetamine in one of a variety of contexts or tested in a variety of contexts for locomotor sensitization. It is clear that conditioning in a novel context, sometimes called "third world" environment, elicits better locomotor sensitization than repeatedly administered drug in a rat's familiar home cage (Badiani et al., 1995; Anagnostaras and Robinson, 1996; Badiani et al., 2000; Anagnostaras et al., 2002). It is hypothesized that the increased glutamate released in response to a novel environment allows for greater sensitization to amphetamine at lower administration and test doses.

Regardless of the mechanisms, the result of this novel-context conditioning is the expression of locomotor sensitization only when rats are acutely challenged with a drug injection in the same context in which they were conditioned. Understanding the

mechanisms encoding context-specific locomotor sensitization can provide input to future therapeutic developments for drug addiction.

4. Anatomy of behavioral sensitization

Because stimulants, such as amphetamine, that induce behavioral sensitization are known to influence the tone of dopamine in the brain by altering dopamine release at the transporter, extensive research has been done on the midbrain dopamanergic system to understand learned associations (Kalivas, 2004). Though other receptor systems in the brain have been affiliated with amphetamine sensitization (Everitt and Robbins, 2005; Zaniewska et al., 2015), dopamine is the most widely studied.

The midbrain dopamanergic system includes multiple brain regions mirrored across hemispheres, but also encompasses the limbic system. The limbic system is often credited for the function of motivation, learning, and memory. Midbrain limbic areas include amygdala, hippocampus, thalamus, hypothalamus, habenula as well as others. Many of these areas provide input to the basal ganglia. The basal ganglia is a group of subcortical nuclei that connect cortex, thalamus and brainstem, as well as functionally defined subcortical areas. There are two, parallel projection pathways in the subcortical basal ganglia. One circuit known for its involvement in learned movement as well as heavily conditioned reward learning and includes dorsal striatum, globus pallidus and substantia nigra, while new reward learning is canonically considered to occur through ventral striatum, ventral pallidum, and ventral tegmentum (Smith and Kieval, 2000). However, it is noteworthy that both basal ganglia circuits have been shown to play roles in motor-action planning, decision-making, learned-reinforcement, and motivation, and other cognitive behaviors.

The majority of brain dopamine, which is projected vastly to areas of the midbrain dopamanergic system, is synthesized in the two small basal ganglia areas, substantia nigra and ventral tegmentum (Luo and Huang, 2016). The striatum is a primary target of these dopamine projections and appropriately rich in G-protein-coupled dopamine receptors. Dorsal striatum is often subdivided into caudate and putamen, with extensive evidence of its involvement in planned motor function and habituated behavior, while ventral striatum is subdivided into nucleus accumbens and olfactory tubercle in rats and humans. The nucleus accumbens is often a targeted site of research for addiction because of its role in reward learning, particularly newer learned associations. Nucleus accumbens and has been proven repeatedly to play a pivotal role in behavioral sensitization, making it a prime region for studying conditioned learning.

4.1. Nucleus accumbens

In rats and humans, each hemisphere of the brain has its own nucleus accumbens comprised of a collection of neurons and described as having an inner core and outer shell. These sub-structures are appropriately named accumbens core and shell differentiated mainly by function. Functional differences are highlighted by afferent projections, efferent projections, and few morphological distinctions in the density and arborization of neurons. Structurally, the accumbens core resides around the anterior commissure, and accumbens shell wraps around the medial-ventral edge of the core. Both regions touch the tip of the lateral ventricle and vary in size and shape in their rostral to caudal assembly. More recently, the shell has been further broken into lateral, medial, and dorsomedial regions based on activity and function, though these distinctions are more nuanced (Reed et al., 2015).

Afferents from limbic regions, such as prefrontal cortex, amygdala, thalamus, and hippocampus are glutamatergic, releasing the excitatory neurotransmitter glutamate, and project to both the core and shell. These afferent areas can be subdivided, and often preferentially innervate the accumbens core or shell differentially by afferent sub-areas. The primary source of dopamine projections to ventral striatum is the ventral tegmentum, which topographically innervates the nucleus accumbens (Clarke and Adermark, 2015).

Efferent projections from the nucleus accumbens are GABAergic, releasing the inhibitory neurotransmitter GABA. These GABAergic outputs mainly project to the thalumus and ventral pallidum with downstream targets in cortical areas known to regulate motor planning and execution. In this way, the nucleus accumbens can indirectly influence afferent brain areas to control the tone of glutamate in the nucleus accumbens. Some of the functional distinctions between nucleus accumbens areas can be understood by the efferent projections of medium spiny neurons.

Accumbens core is often considered more similar to the dorsal striatum with known involvement in motor programming of acquisition of learned behaviors. The accumbens shell is thought to more abstractly encode motivational salience and subjective interpretation, such as "liking" (Berridge et al., 2009). Still, some studies of accumbens core and shell find the functional roles of these areas can act in direct opposition for a given behavior (Sellings and Clarke, 2003; Ito et al., 2005; Clarke and Adermark, 2015). Though there are constant discoveries challenging the rigidity of canonical functions attributed to the striatum, generalizations offer some insight to their potential roles encoding learned associations.

It is worth noting that there is amassed evidence that brain systems outside the basal ganglia are also involved in behavioral sensitization and other addiction-related behaviors. Similarly, although dopamine has a clear role modulating addiction and motivation, it is not optimized to induce hedonic learning without glutamate (Badiani et al., 2000; Ostrander et al., 2003). Given the single source of dopamine and multiple sources of glutamate innervating the accumbens, it is logical that glutamate would have clear role in manipulating accumbens signaling and thusly accumbens encoded behavior. With known roles for dopamine and glutamate in the case of behavioral sensitization, cue-dependent plasticity of neurons has been found repeatedly in both accumbens core and shell (Nestler, 2001; Kalivas, 2004).

4.2. Cell-type distribution in the nucleus accumbens

Although accumbens shell and accumbens core are considered distinct sub-areas, they have similar cellular architecture. In both areas, >90% of nucleus accumbens neurons are GABAergic medium spiny neurons acting as the primary output of accumbens. They are named so because of their morphology. They are uniform in size and have extensive dendritic arbors covered in dendritic spines, which are specialized sub-structures designed to expertly integrate glutamatergic and dopaminergic input.

Medium spiny neurons of the nucleus accumbens can be further subdivided into two distinct populations: D1-receptor-expressing and D2-receptor-expressing (Perreault et al., 2010). They are G_o- and G_i-coupled proteins, respectively, acting through different cell signaling processes in relative-opposition. In normal working brains, different signaling pathways of these dopamine-receptors are found to play complimentary roles acting in opposition, but both allowing for bi-directional plasticity;

they are not restricted to unidirectional plasticity (Shen et al., 2008). They are the two most extensively studied dopamine-receptors in addiction, with younger studies accounting for expression and roles of D3-, D4-, and D5-receptors (Adinoff, 2004; Sun et al., 2016). In the accumbens, approximately half the neurons are shown to be D1-receptor-expressing, while the other half are D2-receptor-expressing, with few instances of receptor co-expression (Perreault et al., 2010; Thibault et al., 2013). D1- and D2-receptor subtypes are found in the nucleus accumbens to co-express enkephalin or dynorphin, respectively (Steiner and Gerfen, 1998; Smith et al., 2013). Though GABA is the primary output of the medium spiny neurons, these unique opioid receptors ligands co-release on to target brain regions allowing for differential messaging post-synaptically (Steiner and Gerfen, 1998). Though less evidence for divergence of D1- and D2-receptor-expressing pathways is found in for in nucleus accumbens than dorsal striatum "direct" and "indirect" signaling pathways (Smith et al., 2013), these distinct dopamine-receptor-expressing neurons of accumbens are thought to have somewhat divergent roles in addiction.

Recent studies have shown that D1- and D2-receptor-expressing neurons have antagonistic effects on drug related behaviors. In some studies activation of D1-receptor-expressing neurons and inactivation of D2-receptor-expressing neurons show enhanced sensitivity to drugs (Lobo et al., 2010) or the reverse (Yoon et al., 2016), most likely dependent on drug, dose, and timing (Kai et al., 2015). In some cases different results have been seen for rats and mice in dopamine-receptor responses ((al-Tajir et al., 1990; Ralph-Williams et al., 2003; Mattson et al., 2007a; Smith et al., 2013), and it is thought glutamate modulation at synapses of medium spiny neurons is

differentially regulated for D1- and D2- receptor neurons (Surmeier et al., 2007). Though findings with amphetamine sensitization have indicated that the expression of different dopamine-receptors, by measuring associated dynorphin and enkephalin proteins, indicate equal recruitment of D1- and D2-receptor-expressing neurons of the striatum (Jaber et al., 1995; Mattson et al., 2007a).

However, medium spiny neurons do not simply provide efferent projections. There is some lateral regulation between medium spiny neurons, though primary inhibition of nucleus accumbens output comes from the remaining, diverse population of interneurons (Clarke and Adermark, 2015).

Each of the interneuron subtypes make up a total of 5-10% of accumbens neurons, with each subtype claiming ≤1% of the total accumbens makeup. Morphologically contrasting medium spiny neurons are striatal cholinergic interneurons. Though small in number, these neurons are large, aspiny cells with extensive axonal branching heavily influencing striatal function. They play a crucial role in the depolarization of medium spiny neurons by acetylcholine, with fewer projections found to other interneurons (Lim et al., 2014). The remaining heterogeneous population of GABAergic interneurons potentiates medium spiny neurons with strong inhibitory innervation. There are various distinct populations of GABAergic interneurons categorized by differences in spike rate and spike threshold. Conventional subtypes of GABAergic interneurons are delineated by protein expression, for example expression of parvalbumin, neruopeptide Y, nitric oxide synthase, somatostatin, calretinin and others (Clarke and Adermark, 2015).

In addition to studying cell-type roles and diversity in the accumbens, expression of many transient factors, such as immediate early genes, for example ΔFosB, ERK, Fos, Zif268, Egr-1, to name a few, have also been found after chronic drug use (Hope et al., 1994; Crombag et al., 2002; Lobo et al., 2010). Many of these transient makers, particularly the Fos-family proteins, are found across cell subtypes after behavioral sensitization and reliably mark activated neurons.

5. Neuronal ensembles

There is a high-degree of resolution needed to encode learned associations between rewarding effects of drugs and complex cues of stimuli. Initial attempts to understand the encoding mechanisms of learned associations were primarily done by analysis of whole brain area, and later, when possible, divided by cell subtypes as just explained for the nucleus accumbens. However, neither of these methods provides enough resolution or required subtlety to encode the dynamic number of complex behaviors rats and humans can exhibit through learned associations. Adaptations of a theory first proposed mid-20th century by Donald Hebb has transformed into the more modern neuronal ensemble hypothesis. It theorizes that learned associations are encoded by sparsely distributed, synchronously activated neurons (Cruz et al., 2015).

Initial studies of activated neurons were done with single-unit recordings, and showed support for the role of neuronal ensembles to encode addiction-related learning. Recordings of firing rate in response to addiction models, such as self-administration (Cruz et al., 2015) and conditioned place preference (Faust et al., 2013), revealed phasic firing of accumbens neurons in response to a variety of rewards, with a transition

of phasic firing occurring instead for cues and context of drug-associated stimuli, even when drug is not on board.

5.1. Fos-expressing neuronal ensembles

Within the last two decades, advances in rodent genetics and genetic manipulation, have provided the methods to more selectively observe and target neurons activated during behavior (Cruz et al., 2015). New, successful methodologies for creating transgenic rats and viral vectors have provided a unique opportunity to study specifically-activated neuronal ensembles, and test if and how they are encoding addiction-related behavior. The best candidates for time-locked identification of specifically activated neurons are transiently activated immediate early genes known to be transcriptionally up-regulated after behavior changes in activated neurons, for example, *Fos*, *zif268*, and *arc (Lobo et al., 2010)*. Immediate early gene promoters, which offer low baseline transcription and fast onset of transcription after cell hyperpolarization, are ideal for studying neuronal ensembles by promoting designer genes that can be carried in viral vectors and microinjected into the brain or transgenic rat models.

Though a variety of promoters for immediate early genes exist to test neuronal ensembles, the Fos-promoter is the obvious candidate for expressing exogenous genes to identify and manipulate neurons. For many reasons, including ease of labeling, Fos is the most widely used marker of neuronal activation in the literature. As such, its promoter is well understood. It is activated by calcium dependently phosphorylated transcription factors, namely through ERK/MapK signaling. Peak mRNA Fos expression occurs approximately 30 minutes after neuron activation, and Fos protein

expression peaks approximately 90-minutes after cell activation and is degraded within a few hours. It is part of the Fos-family of proteins, which include the immediate early genes Fra-1, Fra-2, FosB and ΔFosB (Chen et al., 1997). Of these, ΔFosB has some distinct features from Fos.

ΔFosB is an alternatively spliced variant of the FosB gene, which accumulates after repeated drug exposure or stress. This splice variant is missing two degredation domains in the C-terminus, and is phosphorylated at the N-terminus providing high protein stability. Its half-life is approximated to be 9-days and has been found at time points several weeks after neuron activation. After repeated activation of nucleus accumbens neurons by drugs of abuse, ΔFosB becomes the predominant Fos-like protein (Nestler, 2012). These contrasting factors make Fos a great marker for acute activation, and ΔFosB an optimal marker for previously repeated activation. Additionally, expression of Fos and ΔFosB was shown to be increased in response to a variety of behavioral sensitization protocols (Winstanley et al., 2009; Beloate et al., 2016b; Carneiro de Oliveira et al., 2016), and possibly species specific (Conversi et al., 2011). However, it has been shown manipulations and treatments after sensitization conditioning that blocked behavioral sensitization output did not affect ΔFosB expression (Beloate et al., 2016b). This indicated ΔFosB was induced by activity prior to an acute amphetamine challenge injection and not sensitization expression on test day.

5.2. Transgenic rats to study Fos-expressing neuronal ensembles

Development of two major transgenic rat lines that utilize the *Fos*-promoter have opened the door for studying neuronal ensembles. First, the Fos-GFP transgenic rat,

which contains a transgene encoding for a fluorescent FosGFP fusion protein, has allowed the uncovering of unique electrophysiological and molecular alterations of Fos-expressing neuronal ensembles encoding learned associations to reward. Second, the Fos-LacZ transgenic rat, which contains a transgene encoding for the enzyme β-galactosidase, have allowed for manipulation of Fos-expressing neuronal ensembles, as well as labeling with X-gal labeling.

In Fos-LacZ transgenic rats, expression of β-galactosidase is inert to endogenous substrates, but can cleave the pro-drug Daun02 inducing cell apoptosis. In the Daun02 inactivation procedure, neurons provoked to hyperpolarize on an "induction day test," activate the *Fos*-promoter. Approximately 90-minutes after peak induction, inter-cranial microinjections of Daun02 into target brain areas result in ablation of suspected Fos-expressing neuronal ensembles (Smith and Aston-Jones, 2009; Koya et al., 2016). Using this technique, experiments to determine the causal role of Fos-expressing neuronal ensembles on learned drug-associations and behavior have shown attenuation of addiction-related behavior after Daun02 inactivation (Fanous et al., 2012; Cruz et al., 2014; Warren et al., 2016; Whitaker et al., 2017).

More specifically, in cocaine-induced locomotor sensitization, Daun02 inactivation of Fos-expressing neuronal ensembles indicated these cells played a causal role. This was tested again in a context-specific manner, where rats were trained in locomotor chambers with repeated cocaine or saline, given 1-week withdrawal, and after an induction day session either in the locomotor chamber or novel context, microinjected with Daun02 or vehicle in the nucleus accumbens. Later, a locomotor test with acute cocaine injections showed attenuation of the locomotor sensitization in a

context-specific manner, where rats induced in the locomotor chamber had reduced sensitization compared to rats induced in the novel context (Koya et al., 2009).

These Fos-expressing neuronal ensembles with a known causal role in addiction-related behaviors have been found to be limited to 1-5% of neurons in studied brain areas. The small number and distinct patterns of Fos activation easily permit Fos-expressing neuronal ensembles the resolution needed to encode a variety of learned associations (Koya et al., 2009; Whitaker et al., 2016).

6. Experimental summary and hypothesis

For the experiments of this thesis, we studied learned associations of locomotor sensitization by pairing contexts with repeated amphetamine administration. We expected changes in Fos expression throughout the nucleus accumbens in response to locomotor sensitization, and characterized this population by (i) analyzing potential differences of Fos expression in the shell and core, (ii) determining recruitment of nucleus accumbens dopamine-receptor expressing subtypes to the Fos-expressing ensemble, (iii) determining the overlap of ΔFosB-expressing neurons labeled by activation during sensitization conditioning with repeated administration of amphetamine and Fos-expressing neuronal ensembles we believe are encoding behavior. We designed control experiments to prove context-specificity of our locomotor sensitization protocol, and eventually evaluated the causal role these Fos-expressing neurons play in context-specific locomotor sensitization to amphetamine with Daun02 inactivation. Ultimately, we propose that Fos-expressing neuronal ensembles of the nucleus accumbens encoding context-specific sensitization are playing a causal role in amphetamine-induced locomotor activity.

CHAPTER 1: Methods

1. Subjects

Male Long-Evans rats (Charles River, Raleigh, NC), weighing 300-600g, were used for most experiments. Where indicated, certain experiments required use of male Fos-GFP transgenic rats bred in a Long-Evans background, while other experiments utilized male FosLacZ transgenic rats bred in a Sprague-Dawley background. All breeding was done at NIDA, BRC (Baltimore, MD) by NIDA OTTC (Vautier, 2017).

Behavioral experiments lasted 3-4 weeks. All rats were watered and fed ad libitum in standard sized home cages. They were housed on a 12-hour reverse-light cycle (8am-8pm), and experiments were conducted during the active (dark) cycle. Rats were group housed with cage-mates (2-3 rats) undergoing the same repeated drug administration protocol. With few exceptions (veterinary recommendation, unexpected death of a cage mate mid-study, etc.) rats remained pair housed for the duration of the experiment. Rats were acclimated to these housing conditions for a minimum of 7 days prior to drug treatments. All rats not sacrificed for histology were humanely euthanized.

Some rats run in behavior tests described below were excluded from analyses if they died unexpectedly, were flagged unfit for experiments by veterinary staff, weighed over 600g and locomotor activity scores were impeded (bellies dragged, injections could not be guaranteed i.p.), were tested in locomotor chambers exhibiting equipment malfunctions (errors in beam break recordings, etc.), or found post-mortem to have brain infections or cysts.

2. Facilities

All experiments were conducted at the NIDA BRC under the guidelines of the NIH and ALAAC regulations in accordance with NIDA animal safety protocol ASP14-BNRB-

180. Facility temperatures were maintained at 70℃ with 80% humidity. Experiments and surgeries were conducted in separate rooms from housing in the animal facility.

3. Drug treatment and behavioral assays

Amphetamine (d-amphetamine) was purchased from Sigma-Aldrich (cat# 51-63-8) and diluted in 0.9% sterile, injectable saline.

3.1. Experiment 1: Repeated drug administration protocol for locomotor sensitization

To initiate experiments, rats were given 5-days of conditioning. Each day rats were taken from their home cage and administered an intraperitoneal (i.p.) injection of amphetamine (2 mg/kg; n = 25) or saline vehicle (1 ml/kg; n = 23) and immediately placed into dimly lit locomotor activity chamber for 120 minutes (for timeline see Chapter 2, Figure 1A). The locomotor activity chamber (Med Associates, ENV-515-43x43x43cm) was lined with infrared photo-beams used to monitor the location of the rat in the chamber, as measured by two adjacent beam breaks. Activity Monitor Software (Med Associates, VT) was programmed to convert beam breaks into distance traveled, which was used to determine the rats' locomotor activity scores.

Following the last day of repeated drug administration, rats underwent one week of abstinence in their home cage, during which no drug was administered. Finally, on test day, rats were brought to the experiment room. Here, rats from each repeated drug administration group were acutely injected (i.p.) with amphetamine (0, 1, 2 mg/kg; amphetamine, n = 8, 9, 8; saline, n = 7, 9, 7) and immediately placed in the locomotor chamber for 90-minutes to measure their locomotor activity. On a subset of rats

sampled from each test group, restrictive stereotypy was manually scored during testing.

3.2. Experiment 2: Protein expression after locomotor sensitization

Rats were repeatedly administered amphetamine (2 mg/kg) or saline (1 ml/kg) in locomotor activity chambers once daily for 5 days, as described in Experiment 1. Following the last day of repeated drug administration, rats underwent one week of abstinence in their home cage. Finally, on test day, rats were brought to the experiment room. Here, rats from each repeated drug administration group were acutely injected (i.p.) with amphetamine (0, 1 mg/kg; n = 10, each group) and immediately placed in the locomotor chamber. To capture maximal Fos expression (Mugnaini et al., 1989), at the end of the 90-minute test session, rats were deeply anesthetized (isofluorane) and perfused transcardially with 150 mL 0.1 M phosphate-buffered saline (PBS) followed by 500 mL 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) (PFA). Perfused brains were removed and post-fixed in 4% PFA overnight and transferred to 30% sucrose in PBS (pH 7.4) at 4 ℃ until brains sank (approximately 3 days). Brains were then flash-frozen in powder dry ice and stored at -20 ℃ until sectioning for Fos immunohistochemistry. In a separate experiment, 14 additional rats were added to the experiment, and grouped to receive conditioning (amphetamine or saline), testing (0 or 1 mg/kg amphetamine), and brain tissue preparation exactly as outlined above, before being utilized for ΔFosB immunohistochemistry.

3.3. Experiment 3: mRNA expression after locomotor sensitization

Rats were repeatedly administered amphetamine (2 mg/kg; n = 6) or saline (1 ml/kg; n = 4) and followed by 1-week abstinence, exactly as described above in

Experiment 1. Due to resource constraints, we limited our acute injection on test day to 1 mg/kg, prioritizing the observation of rats exhibiting locomotor sensitization while controlling for potential effects of acute amphetamine. Test sessions were truncated to 30-minutes, to allow for sacrifice at peak *Fos* mRNA expression, and rats were heavily anesthetized with isoflurane for live decapitation. Fresh brains were carefully removed and snap-frozen for 20 seconds in -50°C isopentane, and stored in the – 80 °C freezer until ready for further processing.

3.4. Experiment 4: Protein expression after locomotor sensitization in Fos-GFP transgenic rats

Transgenic Fos-GFP rats were conditioned through the entire amphetamine sensitization protocol described in Experiment 2, with the same fixation protocol. Brains were stored in foil wrapped tubes to prevent fluorescence bleaching of GFP until they were flash-frozen with powdered dry ice. They were then stored at -20 °C until further processed for immunofluorescent colabeling of FosGFP and ΔFosB.

3.5. Experiment 5: Repeated drug administration protocol for context-specific locomotor sensitization

To determine if the locomotor activity and histology obtained through our sensitization protocol was due to context-specific learned-associations or pharmacological effects of amphetamine, we designed a second context (context B) in which rats could be sensitized (for timeline see Chapter 2, Figure 5A).

In an attempt to reduce potential interference of novelty exploration on locomotor activity recorded test day from rats sensitized in context B, all rats were given a 2-hour habituation to the test locomotor chambers (context A) prior to sensitization

conditioning. During sensitization conditioning rats were removed from the home cage and, over 5 x 2-hour sessions, rats were repeatedly administered amphetamine or saline (i.p.) in context A (2 mg/kg amphetamine, n = 27; saline, n = 28) or context B (2 mg/kg amphetamine, n = 23; saline, n = 21). Context B chambers were large microinjection bowls (Harvard Apparatus, CMA830931, 36cm high x 30cm diameter) with 150 g of woodchip bedding and small plastic toy; lights were turned off and music (Mix, 2002) was played continuously in the background.

On test day, after 1-week abstinence in the home cages, all rats were given an acute injection amphetamine (0, 1 mg/kg) and immediately placed in context A. Rats from different conditioning groups were distributed evenly (for n per group see section 2. Figure 6B). At the end of the 90-minute test session, rats were heavily anesthetized, transcardially perfused, and brains stored as described in Experiment 2, in preparation for Fos and ΔFosB immunohistochemistry.

3.6. Experiment 6: Repeated drug administration protocol after context-specific locomotor sensitization in Fos-GFP transgenic rats

Fos-GFP transgenic rats were sensitized to repeated amphetamine in different contexts, and tested for sensitization. Similar to Experiment 5, rats were given a 2-hour habituation to the testing chamber (context A) prior to sensitization conditioning to reduce interference of novelty exploration on test day (for schematic see Chapter 2, Figure 6A). Fos-GFP rats received 5 sessions of repeated administration of amphetamine (2 mg/kg) in context A (n = 7), context B (n = 7), or the home cage (n = 3). Rats sensitized in context A and B were returned to the home cage after 2-hours. Following 1-week abstinence in the home cage, all rats received an acute injection

amphetamine (1 mg/kg) prior to a 90-minute locomotor activity test in context A. Immediately after testing rats were anesthetized, transcardially perfused, and brains stored as described in Experiment 4, in preparation for FosGFP and ΔFosB immunofluorescent colabeling.

3.7. Experiment 7: Daun02 inactivation after context-specific locomotor sensitization in Fos-LacZ transgenic rats

To use the Daun02 inactivation procedure in assessing the causal role of Fos-expressing neuronal ensembles on context-specific locomotor sensitization, modifications to Experiment 5 were made (for schematic see Chapter 2, Figure 7A). First, the Daun02 inactivation procedure required the implantation of guide cannula (23 gauge; Plastics One) bilaterally, 1 mm above the nucleus accumbens. We anesthetized FosLacZ transgenic rats with isofluorane and secured them in a Kopf digital-stereotax. The nose bar was set at -3.3 mm, and the coordinates used to target the nucleus accumbens shell were anteroposterior +1.6, mediolateral ± 2.3 (10 ° angle), and dorsoventral -7.5 mm from rats' Bregma. Cannula were permanently fixed to the rat's skull with dental cement and jeweler's screws. To reduce pain during recovery, rats were injected with Ketoprofen (2.5 mg/kg, sub cutaneous) for up to 3 days post-surgery. Rats were given at least one week to fully recover before beginning sensitization.

Experimental procedures for sensitization conditioning were exactly the same as Experiment 5 including context A habituation, 5 x 2-hour sessions of repeated amphetamine or saline administration in context A (amphetamine, n = 20; saline n = 10) or context B (amphetamine, n = 13; saline, n = 9). Home cage abstinence period

was reduced to 6-days to allow for a Fos-induction session on day 6 after the last session of sensitization conditioning.

On Fos-induction day, all rats were removed from the home cage and acutely injected (i.p.) with 1 mg/kg amphetamine immediately before a 90-minute locomotor activity test in context A. Following Fos-induction sessions, rats from different conditioning groups were microinjected in the testing room with either Daun02 (Sequoia Research Products; dissolved in vehicle) or vehicle (5% DMSO, 6% Tween 80, 89% 0.1 M PBS). Microinjections lasted 1 minute at a rate of 0.5 µg/0.5 µL/side using a syringe pump (Harvard Apparatus) with 10 µL Hamilton syringes attached via polyethylene-50 tubing to 30 gauge injectors (Plastics One) that extend 1 mm beyond the guide cannula into accumbens shell. Injectors were left in place for 1-minute before removal, and returning the rat to the home cage.

Three days after the Daun02 inactivation or vehicle microinjections on Fos-induction day, all rats were tested for locomotor activity in context A for 90-minutes immediately following an acute injection of 1 mg/kg amphetamine (i.p.). Immediately following the test session, rats were deeply anesthetized, trandscardially perfused, and brains stored exactly as described in Experiment 2, in preparation for X-gal labeling.

4. Histology

For all immuno- labeling sections were kept free floating, all wash steps lasted 10 minutes and were done in triplicate, and all washes and incubations were conducted on a gentle plate shaker in solutions with pH 7.4 at room temperature, unless otherwise stated.

4.1. Immunohistochemistry labeling

Whole, fixed brains from rats in Experiment 2 and 5 stored at -20 ºC for immunohistochemistry were coronally sliced (40 µm sections) for nucleus accumbens (~+1.32 to +1.6 from Bregma) using a cryostat (Leica, CM1850 UV; -15 °C), and stored free floating in PBS-azide (1 M PBS, 0.01% azide, pH 7.4) in a 4 ºC refrigerator.

4.1.1. Fos immunohistochemistry

For Fos immunohistochemistry, 3-6 accumbens sections per rat were plated, and sections were washed in PBS. Sections were then transferred to blocking buffer (3% normal goat serum (NGS), 0.25% Triton X-100 in PBS (PBS-Tx)) for 1-hour, and next, incubated over night at 4ºC with anti-Fos antibody diluted in blocking buffer (1:16000 dilution; Cell Signaling Technology, #2250). The following day, sections were washed in PBS, and transferred to biotinylated goat anti-rabbit secondary antibody (1:600 dilution; 1% NGS, in PBS-Tx) for 2-hours. Sections were, again, washed in PBS, and incubated in avidin-biotin-peroxidase complex (Vector Laboratories, ABC Elite kit, PK-6100) in PBS. Next, sections were triple washed in PBS, and developed in 3,3'-diaminobenzidine (DAB) for approximately 4-minutes, rinsed in PBS and mounted onto chrom-alum/gelatin-coated slides. Once dry, slides were dehydrated through a graded series of alcohol (30, 60, 90, 95, 100, 100% ethanol) and cleared with Citrasolv (Fisher Scientific) before coverslipping with Permount media (Sigma-Aldrich).

4.1.2. *ΔFosB immunohistochemistry*

For ΔFosB immunohistochemistry the exact same protocol as described above in section 1.4.1.1. for Fos immunohistochemistry was used, replacing the anti-Fos

antibody with anti-ΔFosB antibody in blocking buffer (1:100,000 dilution; Cell Signaling Technology, #2263).

4.2. Dual-immunofluorescence labeling

4.2.1. *Fos and NeuN co-immunofluorescence*

For a subset of rats in Experiment 2 (n = 7) that were amphetamine sensitized and injected with 1 mg/kg amphetamine on test day, a set of nucleus accumbens brain sections were fluorescently labeled for anti-Fos and anti-NeuN antibodies to determine the percentage of Fos-expressing neurons in the nucleus accumbens.

For Fos and NeuN dual-immunofluorescence labeling, 2-4 accumbens sections per rat were plated, and sections were washed in Tris-buffered saline (TBS) (0.025 M Tris-HCl, 0.5 M NaCl, pH 7.4). Sections were then incubated for 20 minutes in 0.2% Triton X-100 in TBS (TBS-Tx) before 48-hour dual-incubation at 4 ºC with anti-Fos antibody (1:2000 dilution) and anti-NeuN antibody (1:2000 dilution; Chemicon) diluted in TBS-Tx. Next, slices were washed in TBS, and all remaining steps were done in the dark. Dual incubation with Alexa-488 anti-mouse secondary antibody (1:200 dilution; Jackson Labs) to label Fos, and Alexa 568 anti-rabbit secondary antibody diluted in TBS-Tx (1:200 dilution; Jackson Labs) to label NeuN lasted 1-hour. Sections were given a final wash in TBS, and mounted on chrom-alum/gelatin-coated slides. Slides were coverslipped with Mowiol mounting solution (24% glycerol, 9.6% Mowiol, 2.5% DABCO, diluted in 50% TBS (pH 8), and 25% distilled water) and stored at 4 ºC.

4.2.2. *Fos and FosGFP co-immunofluorescence*

Whole, fixed brains from stored at -20 ºC for immunofluorescent labeling were coronally sliced for nucleus accumbens (40 µm sections) using a cryostat. For Fos-GFP

transgenic rats used in Experiments 4 and 6, precautions to reduce light exposure were made, and sections were stored free floating in PBS-azide at 4 ºC. Since the anti-Fos and anti-ΔFosB antibodies were created in the same host (rabbit), we utilized the FosGFP fusion protein expressed by FosGFP transgenic rats to label Fos-expressing neurons with anti-GFP antibody.

To verify the same neurons were being labeled by anti-Fos and anti-FosGFP labeling we ran a dual-immunofluorescence labeling protocol exactly as described in section 4.2.1. for Fos/NeuN labeling using a subset of rats (n = 12). We replaced anti-NeuN antibody with anti-GFP antibody (1:2000 dilution; Cell Signaling Technology, #2955). Alexa-488 anti-mouse secondary labeled FosGFP-expressing neurons, and Alexa-568 anti-rabbit labeled Fos-expressing neurons. All procedures for mounting, coverslipping, and storage were the same.

4.2.3. *Fos and ΔFosB co-immunofluorescence*

All rats from Experiments 4 and 6 underwent FosGFP and ΔFosB dual-immunofluorescence labeling. FosGFP/ΔFosB dual-fluorescence-labeling utilized the exact same protocol described above in section 1.4.2.2. used for Fos/FosGFP, replacing the anti-Fos antibody with anti-ΔFosB antibody (1:10,000 dilution).

4.3. RNAscope *in situ* hybridization labeling

Whole, fresh brains from rats in Experiment 3 stored at -80 ºC for RNAscope *in situ* hybridization labeling were slowly thawed to -18 ºC and coronally sliced for nucleus accumbens (16 µm sections) using a cryostat. Generally, 3-5 slices per rat were immediately mounted onto chilled Superfrost Plus Slides (Fisher Scientific, #12-550-15) and kept at -18 °C. Slides were then stored at -80 ºC overnight or until labeling.

For RNAscope *in situ* hybridization labeling, slides were fixed by immersion in chilled 10% Formalin (Fisher Scientific) for 20-minutes at 4 ºC, and transferred to PBS. Sections were dehydrated through a graded series of alcohol (50, 70, 100% ethanol). Full protocol details of washes, protease incubations, and amplification steps was previously described (Rubio et al., 2015). In brief, we used probes (ACDbio) designed to label the *D1-receptor* (*D1-r*; ACDbio, Rn-DrD1a), *D2-receptor* (*D2-r*; ACDbio, Rn-Drd2-C2), and *Fos* (ACDbio, Rn-Fos-C3) mRNA in the rat brain. Probes were mixed to a 1x 1:1:1 dilution, and incubated for 2-hours at 40 ºC. After a series of amplification steps, we labeled half the slices with an amplifier to labeled *D1-r* with Alexa 488, *D2-r* with Atto 550, and *Fos* with Atto 647 (Amp4 Alt A). The other half of slices were labeled so *D1-r* and *D2-r* had the inverse label (*D1-r* with Atto 550; *D2-r* with Alexa 488), while *Fos* remained the far-red channel (Amp4 Alt B). Sections were then washed, dried, and coverslipped with Mowiol containing 0.15% DAPI. Slides were stored at 4 ºC.

4.4. X-gal labeling

Whole, fixed brains from rats in Experiment 7 stored at -20 ºC for X-gal labeling were coronally sliced for nucleus accumbens (40 µm) using a cryostat. Slices were stored free-floating in PBS, without azide at 4 ºC until labeleded. As needed, PBS was replaced weekly to prevent contamination of sections.

For X-gal labeling, sections were washed in PBS and incubated in X-gal reaction solution containing the following (in mM): 5 $K_4Fe(CN)_6 \cdot 3H_2)$, 5 $K_3Fe(CN)_6$, and 2.4 X-gal for 4-12 hours at 37 ºC while shaking. Finally, sections were washed again in

PBS before they were mounted, dehydrated, and coverslipped the same as described for immunohistochemistry and labeling in section 4.1.1.

4.5. Imaging

All images were taken using a EXi Aqua camera (QImaging) attached to a Zeiss Axioskop 2 light microscope at 20x magnification. For analysis of nucleus accumbens shell and core, positively labeled-nuclei from the entire image, which captured approximately 2.34 mm^2 brain area, were included in quantification. We used iVision for Macintosh (Biovision; version 4.0.15) to adjust imaging parameters and, when necessary, normalize images. During quantification, slides were coded so experimenter was blind to experimental conditions.

4.5.1. *Brightfield imaging*

For Fos- or ΔFosB-immunolabeling, approximately 2-6 images for each rat were captured as bright-field images and quantified for positively-labeled nuclei. Quantifications were semi-automated using iVision software's segmentation feature. The pixel density threshold for detecting Fos-positive nuclei was chosen to match that of the experimenter-determined manual quantification of a slice, and corroborated by a second experimenter. Fos and ΔFosB counts were averaged so that each rat contributed as *n* of 1 for accumbens shell and core.

4.5.2. *Fluorescence imaging*

For epifluorescence imaging of fluorescently labeled Fos, FosGFP, ΔFosB protein, and RNAscope-labeled mRNA, approximately 2-6 images for each rat were captured and quantified for positively-labeled nuclei. Images were set to a threshold of 50 to reduce background noise for quantification. Counts were manually done for each

fluorescent channel using Fiji software (ImageJ 1.51n). When channels were merged, colabeling of dual-marked cells was determined by overlap of > 70% between channels, and then manually quantified. Counts were averaged so that each rat contributed as *n* of 1 for accumbens shell and core final quantifications.

5. Data analysis

Locomotor activity was converted with Activity Monitor software to Microsoft Excel 2011 files, where it was sorted, organized, and analyzed for descriptive statistics. Statistical analysis, such as Pearson's correlations, un-paired t-tests, and χ-squares were done in GraphPad Prism 7. All other statistical analysis was done in IBM SPSS 23. All ANOVAs (multi-factorial, three-way, two-way, one-way) and ANOVA assumptions tests (for data outliers, normality, homogeneity, linearity, test of sphericity, etc.) were done in IBM SPSS 23 in accordance with *Laerd Statistical Tutorials and Software Guides (Laerd-Statistics, 2017)*. Appendix A contains details and results of assumptions tests.

For Fos and ΔFosB expression, in addition to separate ANOVAs, we also ran a multivariate-ANOVA (MANOVA), which allows us to compare two dependent variables, in this case the protein expression in the accumbens shell and accumbens core, together as a combined variable, measuring protein expression in the overall nucleus accumbens. This requires additional measurement of a Wilk's λ distribution to test for differences in variation of the dependent variables (protein expression) not explained by the independent variables (repeated administration x acute injection); Wilk's λ values greater than 0.05 were accepted.

Where necessary, simple two-way and one-way ANOVAs were carried out with Fisher's least significant difference (LSD) adjustments. Post-hoc tests were chosen based on type comparison. Within-subject comparisons were done with Sidak adjustments, while between-subject tests utilized Bonferroni or LSD adjustments, where noted. We used $p < 0.05$ to determine significant effects, unless otherwise noted. All figures were made in Graphpad Prism 7 and modified in Microsoft Power Point 2011.

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Chapter 2: Results

1. Experiment 1: Amphetamine sensitization induced locomotor sensitization

The goal of this experiment was to determine an effective amphetamine sensitization protocol to later examine Fos-expressing neuronal ensembles that may encode the locomotor sensitization.

1.1. Repeated drug administration effects on conditioning locomotor activity

We observed locomotor activity across 5 sessions after repeated administration of amphetamine or saline, as laid out in our methods (Figure 1A). We found that across all conditioning sessions locomotor activity in rats injected with amphetamine was greater than rats injected with saline (Figure 1B). We statistically confirmed our finding with a two-way repeated measure ANOVA (repeated administration X session number), which yielded a significant interaction of repeated administration and session number [$F_{(4,184)} = 21.77$, $p < 0.001$]. Sidak post-hoc comparisons expectedly revealed a significantly greater locomotor activity each session between rats repeatedly administered amphetamine and rats repeatedly administered saline (all sessions $p < 0.001$).

We next found progressively sensitized locomotor activity to the dose amphetamine (2 mg/kg) use for repeated administration within-subject (Figure 1B). Sidak post-hoc analysis within subject found no significant differences between conditioning sessions when rats were administered saline. However, rats administered amphetamine showed progressive increases in locomotor activity each session from as early as the second session (for all $p < 0.001$). However, based on findings in the literature, these quick sensitization results were more likely pharmacokinetic and not due to learned associations (Robinson and Becker, 1986). Based on findings previously

in the literature (Robinson and Becker, 1986). In order to later test for Fos-expressing ensembles that encode learned associations we continued experiments using repeated administration for 5-days, and 1-week abstinence before testing for locomotor sensitization.

1.2. Locomotor activity totals test day

After rats underwent repeated drug administration and 1-week abstinence, each repeated drug administration group received an acute dose amphetamine (0, 1, 2 mg/kg amphetamine) and locomotor activity was measured in a locomotor chamber (Figure 1C). Group comparisons revealed overall differences between repeated drug administration groups and acute injection groups (Figure 1C). A two-way ANOVA (repeated administration X acute injection) confirmed no significant interaction between repeated drug administration and acute injection, but exposed main effects of repeated administration [$F_{(1,42)} = 17.562, p < 0.001$] and acute injection [$F_{(2,42)} = 29.656, p < 0.001$]. We continued with Bonferroni post-hoc adjustments to conservatively assess the most robust effects of sensitization between these factors.

Initial post-hoc examination revealed no differences between the different drug administration groups after 0mg/kg acute amphetamine injection. This indicated our sensitization conditioning protocol did not elicit conditioned locomotion, a sensitized anticipatory response occasionally seen after amphetamine sensitized (Robinson and Becker, 1986). This allowed us to use the acute injection of 0 mg/kg amphetamine as a baseline for locomotor activity for each repeated drug administration group from which we made post-hoc comparisons with higher doses of amphetamine.

For rats repeatedly administered amphetamine, locomotor activity was compared between rats acutely injected with 0mg/kg to those acutely injected with 1 mg/kg or 2 mg/kg amphetamine. Post-hoc analysis revealed significant increases in locomotor activity at both doses (both, $p < 0.001$). We next compared 1 mg/kg to 2 mg/kg amphetamine injection, and found no significant differences, thus requiring further analysis to determine an acute amphetamine injection dose for future experiments.

For rats repeatedly administered saline, locomotor activity was compared between rats acutely injected with 0mg/kg to those acutely injected with 1 mg/kg or 2 mg/kg amphetamine. Post-hoc analysis revealed increased locomotor activity only for rats acutely injected with 2 mg/kg amphetamine ($p = 0.0007$), while 1 mg/kg acute injection was not significantly increased from 0 mg/kg acute injection amphetamine. This finding showed rats acutely injected with 2 mg/kg amphetamine do not need prior amphetamine sensitization to induce a significantly higher locomotor activity than baseline. These pharmacokinetic locomotor effects found at 2 mg/kg amphetamine may confound future ability to analyze effects of learned behavior that induce locomotor sensitization, and require further analysis.

1.3. Binned locomotor activity test day

To further test for nuanced differences between repeated drug administration and acute injection doses, we divided locomotor activity on test day into 18 x 5-minute activity bins and compared rats in different treatment groups (Figure 1D). A three-way mixed model ANOVA (repeated administration x acute injection x activity-bin) was not significant. Because we wanted to determine an acute injection dose that induced

optimal locomotor sensitization for future experiments we carried out a two-way repeated measure ANOVAs (repeated administration x activity-bins) comparing repeated drug administration effects on within-subjected binned locomotor activity for each acute injection dose.

As previously found after measuring total locomotor activity on test day (above), analysis of binned locomotor activity after acute injection with 0 mg/kg found no differences between repeated drug administration groups (Figure 1D, panel 1). There was no significant interaction (repeated administration x activity-bin) or main effect of repeated administration. However, for each repeated drug administration group there was a significant decrease in activity from the beginning to the end of the test session [$F_{(17,2210)} = 7.694$, $p < 0.001$], which likely reflects rats' habituation to the context and reduced novelty exploration at the end of the session. Sidak post-hoc tests indicated both repeated drug administration groups first showed decreases from locomotor activity recorded in the first bin after 30 minutes (amphetamine, bin6, $p = 0.041$; saline, bin6, $p = 0.001$), and this decrease was sustained for the remainder of the session (amphetamine, bin7-18, $p \leq 0.001$; saline, bin 7-18, $p < 0.01$).

For rats acutely injected with 1 mg/kg amphetamine on test day, we found robust locomotor sensitization occurs early in the test session as a result of previous repeated amphetamine administration (Figure 1D, panel 2). After 1 mg/kg acute amphetamine, a two-way ANOVA (repeated administration x acute injection) found no significant interaction, but revealed significant main effects of repeated drug administration [$F_{(1,16)} = 8.763$, $p = 0.009$]. Sidak post-hoc analysis comparing rats previously administered repeated amphetamine or saline, found increased locomotor activity of amphetamine

administered rats in the first 35 minutes of the test session (bin2, $p = 0.009$; bin4, $p = 0.041$; bin5, $p = 0.044$; bin7, $p = 0.030$), as compared to saline administered rats. Rats previously administered repeated amphetamine reduced locomotor activity after 45 minutes, at which point they returned to levels comparable with the rats previously administered saline. The ANOVA also revealed a significant main effect [$F_{(17,272)} = 7.94$, $p < 0.001$], which indicated a decrease in locomotor activity from the beginning to end of the test session. Within-subject, Sidak post-hoc analysis comparing the first recorded bin to all subsequent bins revealed that rats previously administered saline were consistent in their locomotor activity for the duration of the test session, which means rats previously administered repeated amphetamine were driving the significance of the main effect for binned locomotor activity. Rats previously administered amphetamine showed decreases in locomotor activity 1-hour after acute injection with 1 mg/kg amphetamine and the start of the session (bin14-18, $p \leq 0.05$). The decrease in activity at the end of the session offers us a window, in the first hour of testing to measure peak locomotor sensitization effects, and highlights an ideal window for observing Fos expression differences between locomotor sensitized and saline administered rats.

In rats acutely injected with 2 mg/kg acute amphetamine on test day, we found a difference between rats repeatedly administered amphetamine or saline (Figure 1D, panel 3). A two-way ANOVA (repeated administration x activity-bin) showed no significant interaction, and no significant changes in locomotor activity from the beginning to the end of the session. There was a significant main effects of repeated administration [$F_{(1,13)} = 6.947$, $p = 0.021$], which indicated rats previously administered

repeated amphetamine sustained were more active throughout the session than rats previously administered saline. However, Sidak post-hoc test comparing repeated drug administration groups reveal that only once, briefly, towards the end of the test session were there significant differences between repeated amphetamine and repeated saline administration (bin14, $p = 0.047$). The lack of difference between repeated amphetamine and saline administration groups can be explained by the finding in the previous section comparing total locomotor activity test day, that revealed rats previously repeatedly administered saline increased locomotor activity to levels above baseline (0 mg/kg acute injection) when they were injected with 2 mg/kg amphetamine. This effect explains why saline groups were high, however, to explain why rats previously administered repeated amphetamine did not simply exhibit a more sensitized locomotor response, we measured for interference in locomotor activity with sensitization of another behavior, restrictive stereotypy.

1.4. Binned stereotypy scores test day

Restrictive stereotypy, characterized by perseverative movements in sensitized rats can interfere with locomotor activity (Robinson and Becker, 1986). Stereotyped behaviors were assessed every 5 min for 30 s each time using a 9-point rating scale adapted from Ellinwood and Balster (Ellinwood and Balster, 1974)—1: asleep; 2: inactive; 3: normal in place activity; 4: normal, alert, rearing, normal level of locomotor activity; 5: rearing, high level of locomotor activity; 6: slow patterned behaviors, no rearing, normal level of locomotor activity; 7: faster patterned behaviors, no rearing, high level of locomotor activity; 8: highly repetitive patterned behaviors in a restricted area; 9: backing up, abnormally maintained posture; seizure.

In a subset of rats, we tested for interference of restrictive stereotypy across a 90-minute test session (18 x 5-minute stereotypy-bins) (Figure 1E). A three-way mixed model ANOVA (repeated administration x acute injection x stereotypy-bin) showed no interaction. We then ran two-way repeated measure ANOVAs (repeated measure x stereotypy-bin) comparing repeated drug administration effects on within-subjected binned stereotypy scores for each acute injection dose.

Rats from both repeated drug administration groups showed relatively normal range in activity (2-4) throughout the session after acute injection with 0 mg/kg amphetamine, with more low activity bins at the end of the test session (Figure 1E, panel 1). A two-way ANOVA (repeated administration x stereotypy-bin) showed no significant interaction, but revealed main effects of repeated administration [$F_{(1,4)}$ = 21.81, p = 0.01] and lower scores at the end of the test session [$F_{(17,68)}$ = 2.329, p = 0.007]. These findings parallel decreases seen at the end of the test session for locomotor activity, analyzed above. Though rats previously administered repeated amphetamine had scores in a slightly higher range than rats administered repeated saline, there were no individual bins where Sidak post-hoc comparisons found significant differences.

Stereotypy scores for both repeated drug administration groups after 1 mg/kg amphetamine acute injection range from 3-6. Rats previously administered repeated amphetamine occasionally reached stereotypy scores of 7, but never reached scores of 8 or 9. A two-way ANOVA (repeated administration x streotypy-bin) revealed no significant interaction, nor a change in recorded stereotypy scores from beginning to end of session. However, we did find a significant main effect of repeated

administration [$F_{(1,6)} = 0.494$, $p = 0.022$], that was sustained across the session. However, Sidak post-hoc analysis comparing rats between repeated drug administration groups revealed rats have similar stereotypy scores if compared for individual bins. The stereotypy scores recorded reflect the binned locomotor activity analyzed above, where at the same time locomotor activity was at its highest, the stereotypy scores were reaching their highest, 7, in rats previously administered repeated amphetamine in the first 30 minutes of the test session. Regardless, injection with 1 mg/kg acute amphetamine after previous amphetamine sensitization does not result in stereotypic behavior that interferes with locomotor activity.

However, after acute injection with 2 mg/kg amphetamine, rats previously administered repeated amphetamine reach occasional scores of 8, indicating interference with recorded locomotor activity (Figure 1E, panel 3). A two-way ANOVA (repeated administration x stereotypy-bin) revealed no significant interaction, but found main effects of repeated administration [$F_{(1,4)} = 19.08$, $p = 0.012$], as well as a shift in stereotypy scores recorded from the beginning to end of the test session [$F_{(17,68)} = 2.237$, $p = 0.010$]. For the first time, we see significant Sidak post-hoc comparisons between repeated drug administration groups, particularly at the end of the session (bin14, $p = 0.004$; bin 16, $p = 0.004$; bin 18, $p < 0.001$). Reflected in the high locomotor activity analyzed above for rats repeatedly administered saline, acute injection with 2 mg/kg amphetamine does not require prior amphetamine exposure to induce hyper-locomotor activity, as rats in this group had occasional stereotypy scores of 7.

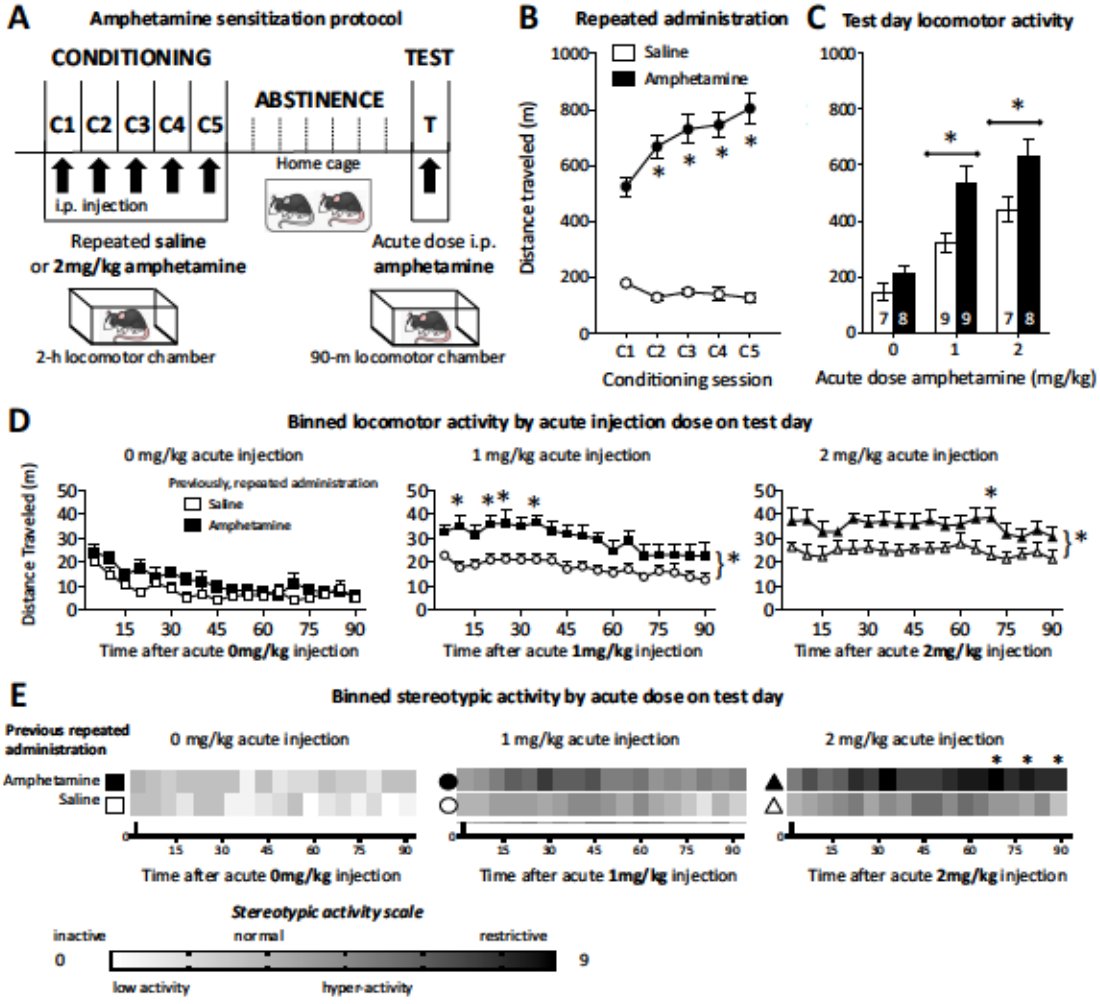
1.5. Summary of amphetamine sensitization protocol effectiveness

In total, these data conclusively show that for our experiments, 1 mg/kg is best for eliciting sensitized locomotor activity in rats previously administered repeated amphetamine. Unlike rats acutely injected with 2 mg/kg amphetamine, after acute injection with 1 mg/kg amphetamine, we did not find pharmacological effects of amphetamine influencing locomotor activity in saline administered rats, nor did we observe restrictive stereotypy in the amphetamine administered rats. At this acute dose, we also revealed a prime window in the first 30 minutes to measure peak differences in locomotor activity between repeated administration groups. This left us with a peak window to measure Fos expression that may be encoding locomotor sensitization.

Figure 1. (on following page)

A) Schematic of repeated drug administration and acute injection protocol to elicit locomotor sensitization in rats. B) Locomotor activity recorded across 5 x 2-hour sessions of repeated administration of saline (white circles) or amphetamine (2 mg/kg; black cirlces). C) Locomotor activity recorded in a 90-minute test when rats previously administered saline (white bars) or amphetamine (black bars) were acutely injected with 0, 1, or 2 mg/kg amphetamine immediately before session. D) Locomotor activity recorded of rats previously administered repeated saline (white) or amphetamine (black) on test day analyzed in 5-minute bins for 3 doses of acute amphetamine injection. E) Stereotypic activity scored in 5-minute bins across test session for a subset of rats (n = 3-4). Stereotypic activity scores in the restrictive range are darkest gray.

Figure 1: Establishing a behavior model and experimental dose for locomotor sensitization



2. Experiment 2: Nucleus accumbens Fos expression after locomotor sensitization

The goal of this experiment was to determine if there are changes in Fos expression in the nucleus accumbens that may encode of locomotor sensitization to amphetamine.

2.1. Locomotor activity

Rats sacrificed for Fos immunohistochemistry exhibited an expected sensitization in locomotor activity when repeatedly administered amphetamine and challenged 1-week later with an acute injection of 1 mg/kg amphetamine (Figure 2A). A two-way ANOVA (repeated administration x acute injection) showed no significant interaction, but revealed significant main effects of repeated administration [$F_{(1,35)} = 6.657$, $p = 0.014$] and acute injection [$F_{(1,35)} = 54.16$, $p < 0.001$]. Bonferroni post-hoc comparisons showed significant increases in locomotor activity of rats repeatedly administered amphetamine and acutely injected with 1 mg/kg amphetamine when compared to rats administered repeated saline ($p = 0.029$) or acute 0 mg/kg amphetamine injection ($p < 0.001$).

2.2. Fos expression in the nucleus accumbens

To measure Fos expression in the nucleus accumbens as a single structure, we combined quantifications for the core and shell and found changes in overall Fos expression (data shown by sub-area only, Figure 2B). We found rats exhibiting sensitized locomotor activity after repeated amphetamine and acute injection with 1 mg/kg amphetamine also had the most Fos expression in the nucleus accumbens. A two-way MANOVA (repeated administration x acute injection) showed no significant interaction, but did yield significant main effects of repeated administration [$F_{(2,34)} =$

5.528[b], p = 0.008, Wilks' λ = 0.755] and acute injection [$F_{(2,34)}$ = 6.299[b], p = 0.005, Wilks' λ = 0.73]. This increase in Fos expression form a Fos-expressing neuronal ensemble, and may reflect activity of nucleus accumbens neurons encoding locomotor sensitization.

A subset of these rats exhibiting sensitized locomotor activity and expressing increased Fos in the accumbens were labeled for Fos/NeuN dual-fluorescence (Figure 2E). Results showed that Fos positive neurons (mean ± SEM: 41.7 ± 10.9) and NeuN positive neurons (720.6 ± 10.6) are colabeled in the nucleus accumbens ~5.8% of the time, indicating our Fos-expressing neuronal ensemble is a very small population of highly activated neurons.

Fos expression in accumbens shell trended in a similar direction to the Fos expression in the combined nucleus accumbens. We found rats exhibiting sensitized locomotor activity after repeated amphetamine and acute injection with 1 mg/kg amphetamine also had the most Fos expression in the accumbens shell (Figure 2C, panel 1). A two-way ANOVA (repeated administration x acute injection) analyzing Fos expression in the accumbens shell revealed significant main effects of repeated administration [$F_{(1,35)}$ = 10.288, p = 0.003] and acute injection [$F_{(1,35)}$ = 7.505, p = 0.01], with no significant interaction. LSD adjusted simple main effects of repeated administration showed there were no Fos expression differences between repeated drug administration groups when injected with 0 mg/kg acute amphetamine, but Fos was significantly increased in amphetamine administered rats with 1 mg/kg acute amphetamine (p = 0.005). Further LSD adjusted simple main effects for acute injection were significant in both repeated drug administration groups (amphetamine, p < 0.001;

saline, $p < 0.001$). These data, in addition to results from a Pearson's correlation between locomotor activity and Fos expression in the shell ($r = 0.354$, $p = 0.027$) indicate a correlation between increased locomotor sensitization and increased Fos expression in the accumbens shell.

Though not always the case, here, we find Fos expression in the accumbens core changes in a similar direction to Fos expression in the shell, and thusly Fos expression in the combined accumbens. We found rats exhibiting sensitized locomotor activity after repeated amphetamine and acute injection with 1 mg/kg amphetamine also had the most Fos expression in the accumbens core (Figure 2C, panel 2). A two-way ANOVA (repeated administration x acute injection) yielded a significant interaction between treatment groups [$F_{(1,35)} = 4.51$, $p = 0.041$]. Bonferroni post-hoc tests revealed a significant increase in Fos expression if rats had previous repeated amphetamine administration before acute injection with 1 mg/kg instead of 0 mg/kg amphetamine ($p < 0.001$) with no differences between acute injection if they were previously administered repeated saline. However, post-hoc analysis revealed no difference repeated drug administration groups for either acute injection group. Similar to the accumbens shell, we find a correlation between locomotor activity and Fos expression in the accumbens core ($r = 0.346$, $p = 0.029$).

Though Fos expression moves in the same direction for accumbens shell and core, on average there are many more activated neurons in the accumbens core (407.8 ± 5.5) than the accumbens shell (188.7 ± 2.1), which may imply activity in response to different information. Additionally, for a subset of rats, we also quantified slightly more posterioir sections of nucleus accumbens (+0.96 to +1.2 mm from Bregma) (Figure

2D). For both shell and core, we found similar effects highlighting the increased Fos expression of rats exhibiting increased locomotor activity (shell: significant interaction [$F_{(1,16)} = 9.606$, $p = 0.007$]; core: significant main effects of repeated administration [$F_{(1,34)} = 5.155$, $p = 0.030$] and acute injection [$F_{(1,34)} = 8.991$, $p = 0.005$]).

2.3. Summary of Fos expression in the nucleus accumbens after locomotor sensitization

Therefore, we conclude that rats exhibiting locomotor sensitization to 1 mg/kg acute amphetamine injection after previous, repeated amphetamine administration have a Fos-expressing neuronal ensemble that appears to be represented in the nucleus accumbens shell and core.

Figure 2: Establishing a Fos ensemble in the nucleus accumbens after locomotor sensitization

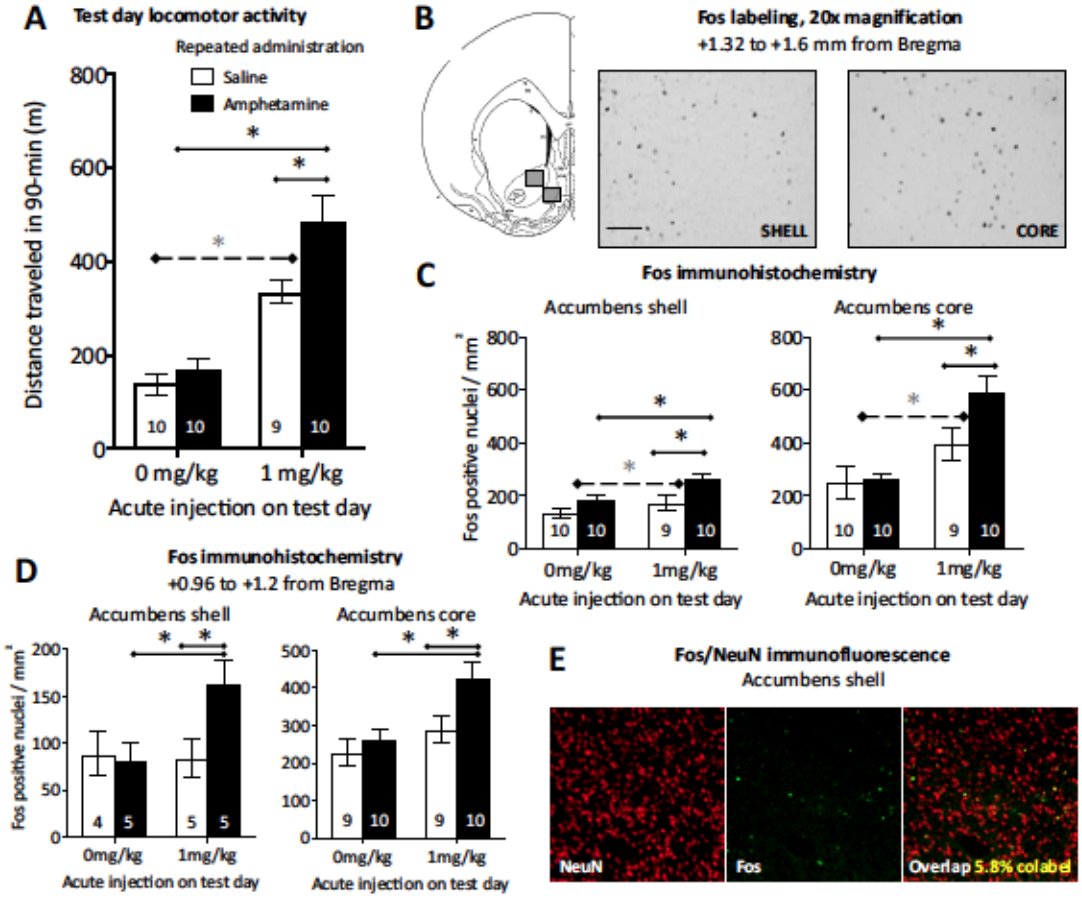


Figure 2.

A) Locomotor activity on test day of rats sacrificed for Fos immunohistochemistry. B) Representative images of Fos-labeling in the nucleus accumbens shell and core. C) Quantifications of Fos-expressing neurons in nucleus accumbens shell and core after locomotor activity testing. D) Fos expression in more posterior areas of nucleus accumbens after locomotor activity testing. E) Representative images of Fos/NeuN immunofluorescent co-expression in nucleus accumbens shell.

3. Experiment 3: *Dopamine-receptor* subtypes recruited to the *Fos*-expressing ensemble

We have established a Fos-expressing neuronal ensemble in the nucleus accumbens correlated to locomotor activity of rats exhibiting locomotor sensitization. By using RNAscope *in situ* hybridization, we observed differences in the distribution of dopamine-receptor-expressing neurons among *Fos*-expressing neurons. Dopamine-receptor-expressing neurons were colabeled with *Fos* for the *D1-receptor* or *D2-receptor* (Figure 3A), or they were found to be non-dopaminergic and express *Fos* only. We aimed to determine what subtypes of dopamine-receptor-expressing nucleus accumbens neurons were recruited to the *Fos*-expressing neuronal ensemble.

To conserve resources, we prioritized the comparison of repeated administration groups. We compared locomotor activity of rats repeatedly administered amphetamine to rats repeatedly administered saline, and both repeated administration groups were acutely injected with 1 mg/kg amphetamine (results not shown). As expected, we found a significant increase in locomotor activity after 1 mg/kg acute amphetamine injection when rats were previously administered repeated amphetamine, as compared to rats administered repeated saline ($t = 2.716$, $df = 8$, $p = 0.026$).

3.1. *Fos*-labeling with RNAscope *in situ* hybridization

Since our previous Fos expression analysis yielded slightly different results between accumbens shell and core above, we first look for a potential interaction of accumbens sub-areas within-subject for Fos expression by RNAscope *in situ* hybridization labeling (Figure 3B).

We found increases in Fos expression for rats previously administered repeated amphetamine compared to rats administered saline when they were acutely injected with 1 mg/kg amphetamine on a 30-minute test session (Figure B, black asterisk). A two-way repeated measure ANOVA (repeated administration x accumbens sub-area) comparing Fos expression did not reveal a significant interaction, nor did it show a main effect of brain area. As expected, however, there was a significant main effect of repeated drug administration [$F_{(1,8)} = 7.533$, $p = 0.025$], which revealed a significant increase in Fos expression for rats repeatedly administered amphetamine. Sidak post-hoc analysis found no pairwise effects. There was, however a significant correlation between locomotor activity and Fos expression in the accumbens shell ($r = 0.66$, $p = 0.039$) but not the core ($r = 0.09$, $p = 0.809$). Unlike with Fos immunohistochemistry, RNAscope *in situ* hybridization labeling did not capture different levels of Fos induction, and means between brain areas were very smiliar. It may be that the thin sections (16 µm) of tissue used for RNAscope *in situ* hybridization or differences in labeling for mRNA instead of protein account for these differences.

3.2. *D1-receptor*- and *D2-receptor*-expressing neurons distribution in nucleus accumbens

Before evaluating receptor distribution in *Fos*-expressing neurons, we first confirmed that RNAscope *in situ* hybridization properly labeled *D1-receptor* and *D2-receptor* neurons in the accumbens shell and core population. The literature indicates that these receptors rarely co-express in the nucleus accumbens, and are evenly divided amongst medium spiny neurons, with 5-10% of neurons expressing neither (Smith et al., 2013; Clarke and Adermark, 2015). We labeled *D1-receptor*- and *D2-receptor*-

expressing neurons with RNAscope *in situ* hybridization amplifiers that were counter-balanced in the *D1*- and *D2*-receptor channel for fluorophore. We next compared the labeling of these two accumbens cell types by accumbens sub-area and found expected distributions of dopamine-receptors. A two-way repeated measure ANOVA (cell type x accumbens sub-area) revealed no significant interactions or main effects. These results confirmed RNAscope *in situ* hybridization as an appropriate labeling method to observe differences in dopamine-receptor subtype distribution with *Fos*-expressing neurons.

3.3. *Fos*-expressing neurons preferentially recruit *D1-receptor*-expressing neurons

We discovered D1-receptor expressing neurons were preferentially recruited to the Fos-expressing neuronal ensembles in the accumbens shell of rats exhibiting locomotor sensitization (Figure 3B). A two-way repeated measure ANOVA (repeated administration x cell subtype) found no significant interaction, nor main effect of repeated administration, but did uncover a significant main effect of cell subtype in *Fos*-expressing neurons [$F_{(2,24)} = 15.53$, $p < 0.001$]. Sidak post-hoc comparisons revealed a significant increase *D1-receptor* co-expression with *Fos* labeled neurons of the accumbens shell of rats repeatedly administered amphetamine, as compared to rats administered saline ($p = 0.012$). All other dopamine-receptor subtypes (*D2-receptor*-expressing and non-dopaminergic) did not significantly differ between repeated drug administration group.

Results from accumbens core were found to be similar to accumbens shell, with increased recruitment of *D1-receptor*-expressing neurons to the *Fos*-expressing neuronal ensemble (Figure 3b). A two-way repeated measure ANOVA (repeated

administration x cell subtype) found no significant interaction, or main effect of repeated administration, but did see a significant main effect of cell subtype in *Fos*-expressing neurons [$F_{(2,24)} = 21.61, p < 0.001$]. Sidak post-hoc comparisons revealed a significant increase *D1-r* co-expression with *Fos* in rats repeatedly administered amphetamine, as compared to rats administered saline ($p = 0.043$). All other dopamine-receptor subtypes (*D2-receptor*-expressing and non-dopaminergic) did not significantly differ between repeated drug administration group.

3.4. Summary of *dopamine-receptor* co-expression with *Fos*-expressing neuronal ensembles

In summary, *D1-receptor*-expressing neurons were the primary neuron recruited to *Fos*-expressing neuronal ensembles of accumbens core and shell in rats exhibiting locomotor sensitization to amphetamine. This unique finding confirms that distribution of neurons recruited to become part of the *Fos*-expressing neuronal ensemble found in the nucleus accumbens after locomotor sensitization to amphetamine is not random or heterogeneous, but instead partial to recruitment of *D1*-r expressing neurons.

Figure 3: Determining cell type distribution in accumbens of Fos-expressing neurons

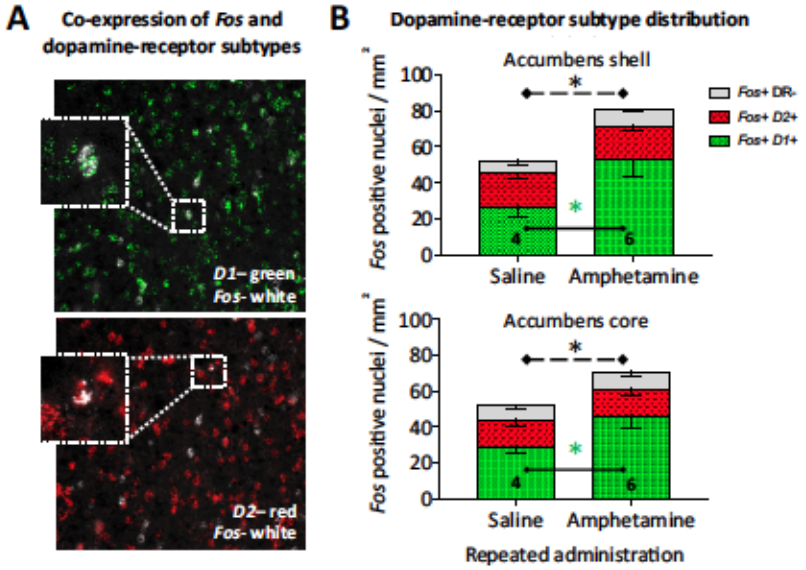


Figure 3.

A) Representative images of mRNA labeling with RNAscope in situ *hybridization for* D1-receptors *(green),* D2-receptors *(red), and* Fos *(white). B) Graph depicting the total number of Fos neurons labeled by RNAscope* in situ *hybridization, indicated by the dashed line and black star, as well as* dopmaine-receptor *subtype distribution within the* Fos-*expressing neruonal population showing significance only in the* D1-receptor *expressing population (green asterisk).*

4. Experiment 4: ΔFosB-expressing neurons were recruited to the Fos-expressing ensemble

We aimed to further characterize the types of neurons being recruited to the Fos-expressing neuronal ensemble found in rats exhibiting locomotor sensitization to amphetamine. We measured the co-expression of Fos and ΔFosB. ΔFosB is a stable protein known to be upregulated with amphetamine sensitization, and here we tested if ΔFosB was a reliable marker of neurons activated during conditioning and then observed co-expression of Fos and ΔFosB in an attempt to understand the relationship between neurons encoding locomotor sensitization and those activated previously during sensitization conditioning.

4.1. ΔFosB as a label for neurons activated during sensitization conditioning to amphetamine

Alternate brain sections from rats that were labeled for Fos expression in Experiment 2 were labeled for ΔFosB immunohistochemistry. These rats showed reliable locomotor sensitization (Figure 4A). A two-way ANOVA (repeated administration x acute injection) revealed a significant interaction (repeated administration x acute injection) [$F_{(1,50)} = 5.023$, $p = 0.029$] on locomotor activity (Figure 4a). Bonferroni post-hoc comparisons show significant increases in locomotor activity of repeatedly administered amphetamine and acutely injected with 1 mg/kg amphetamine when compared to rats administered repeated saline ($p = 0.001$) or acute 0 mg/kg amphetamine injection ($p < 0.001$).

To measure ΔFosB expression in the nucleus accumbens as a single structure, we combined quantifications for the core and shell and found changes in ΔFosB expression

(data shown by sub-area only, Figure 4B). We found increases in ΔFosB expression coincided with previously administered repeated amphetamine with no change in expression as a result of acute injection. A two-way MANOVA (repeated administration x acute injection) showed no significant interaction, nor significant main effects of acute injection, but yielded significant main effects of repeated administration [$F_{(2,50)} = 29.548^{b}$, $p < 0.001$, Wilks' $\lambda = 0.462$]. The increased ΔFosB expression indicates labeling of neuron activity from the conditioning sessions, and reflected in known differences between locomotor activity during conditioning between rats repeatedly administered amphetamine or saline. Therefore, neurons expressing ΔFosB form what we will call a ΔFosB-expressing conditioning ensemble, and can be measured for co-expression with Fos.

Further analysis of the ΔFosB expression in the accumbens shell trended similar to the combined nucleus accumbens. We found rats that were previously administered repeated amphetamine had increased ΔFosB expression regardless of acute injection or locomotor sensitization output (Figure 4C, panel 1). A two-way ANOVA (repeated administration x acute injection) analyzing ΔFosB expression in the accumbens shell revealed significant main effects of repeated administration [$F_{(1,50)} = 33.597$, $p < 0.001$] and no effect of acute injection. LSD adjusted simple main effects of repeated administration showed there were significant ΔFosB expression differences between repeated drug administration groups ($p < 0.001$). Bonferroni post-hoc comparisons between all rats repeated drug administration groups were significantly different no matter the acute injection dose (all, $p \leq 0.02$).

Similar changes in ΔFosB expression were found in the accumbens core as were found in accumbens shell and combined accumbens. We found rats that were previously administered repeated amphetamine had increased ΔFosB expression regardless of acute injection or locomotor sensitization output (Figure 4C, panel 2). A two-way ANOVA (repeated administration x acute injection) analyzing ΔFosB expression in accumbens core revealed significant main effects of repeated administration [$F_{(1,50)} = 36.021$, $p < 0.001$] and no effect of acute injection. LSD adjusted simple main effects of repeated administration showed there were significant ΔFosB-expression differences between repeated drug administration groups ($p < 0.001$). Bonferroni post-hoc comparisons between repeated drug administration groups were significantly different no matter the acute injection dose (all, $p \leq 0.03$).

Together, these data suggest ΔFosB expression in the accumbens shell and core mark neurons that were activated during conditioning, and form a ΔFosB-expression conditioning ensemble. The ability to label neurons that are not acutely induced by amphetamine make this ensemble perfect for labeling neurons activated during conditioning of amphetamine sensitization, and can be compared to activated neurons expressing Fos after locomotor sensitization on test day.

Figure 4: Establishing a neuronal ensemble for sensitization conditioning with ΔFosB expression

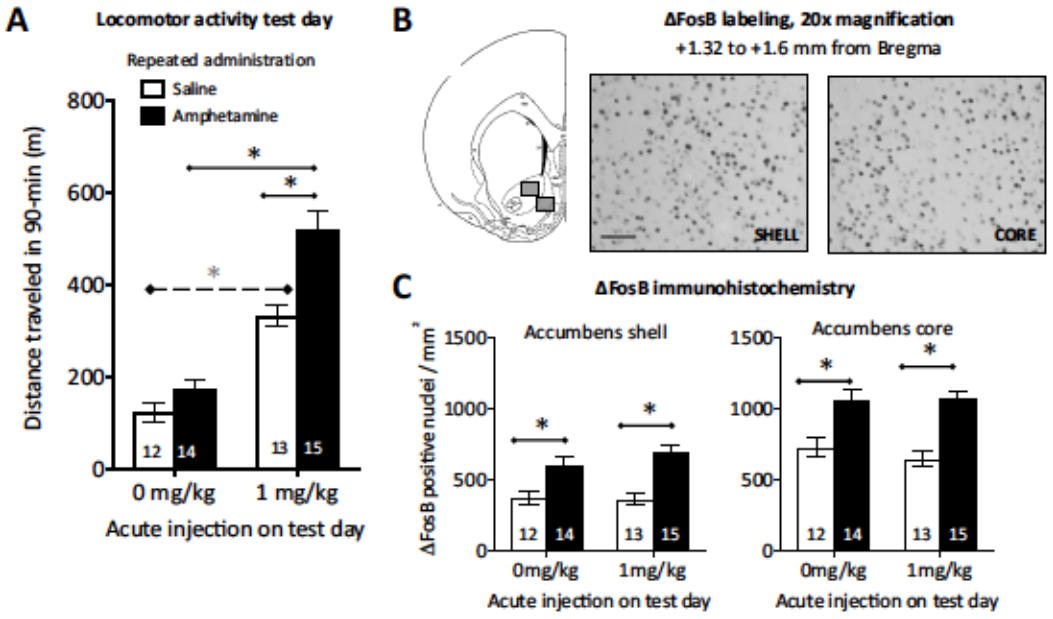


Figure 4.

A) Locomotor activity of rats sacrificed for ΔFosB immunohistochemistry. B) Representative images of ΔFosB immunohistochemistry for accumbens shell and core. C) ΔFosB positive neurons quantified in the nucleus accumbens core and shell after amphetamine sensitization treatment and measured locomotor activity.

4.2. Fos/ΔFosB co-expression after locomotor sensitization

We showed that we could induce and measure neurons activated on test day by labeling the transient protein Fos, and that we could label neurons activated by repeated amphetamine administration by labeling the stable protein ΔFosB. However, to look at the co-expression of Fos and ΔFosB and determine recruitment of neurons activated during conditioning of amphetamine sensitization with neurons activated in rats exhibiting locomotor sensitization on test day in the same brain section, we needed to adjust our antibody labels of these neurons, as both antibodies that have been used for these experiments required an anti-rabbit secondary. Therefore, in these experiments we made use of the Fos-GFP transgenic rat contained a transgene that allowed for expression of FosGFP under a modified Fos promoter (Figure 5A). The goal was to substitute the Fos-labeling antibody with a FosGFP labeling antibody in order to look at co-expression of Fos-expressing neurons and ΔFosB-expressing neurons.

To confirm the substitution of FosGFP-labeling by anti-GFP antibody we measured co-expression of Fos and FosGFP in the accumbens of Fos-GFP transgenic rats. We sensitized Fos-GFP transgenic rats with our established protocol and found that using an anti-GFP antibody and our standard anti-Fos antibody, across all treatment groups colabeling of FosGFP-expressing neurons (107 ± 13.3) and Fos-expressing neurons (102.5 ± 13.0) yielded approximately 95% overlap (Figure 5B). Near perfect co-expression of Fos with FosGFP made it a suitable replacement for future experiments. Next, we examined co-expression of FosGFP and ΔFosB and found increased recruitment of ΔFosB-expressing neurons to the Fos-expressing neuronal ensemble in rats exhibiting locomotor sensitization (Figure 5C).

A further look at the locomotor activity of the amphetamine sensitized Fos-GFP transgenic rats indicated normal sensitization behavior (Figure 5D). A two-way ANOVA (repeated administration x acute injection) on the locomotor activity of Fos-GFP transgenic rats revealed no interaction, but showed significant main effects of repeated administration [$F_{(1,20)} = 13.14$, $p = 0.002$] and acute injection [$F_{(1,20)} = 14.585$, $p = 0.001$]. As previously seen, Bonferroni post-hoc comparisons showed significant increases in locomotor activity of amphetamine sensitized rats acutely injected with 1 mg/kg amphetamine when compared to rats administered repeated saline ($p = 0.036$) or acutely injected with 1 mg/kg amphetamine ($p = 0.038$).

Next, we measured FosGFP expression in the nucleus accumbens of these rats and found increased effects of acute 1 mg/kg amphetamine injection that did not relate to locomotor sensitization (Figure 5E). A two-way ANOVA (repeated administration x acute injection) for quantification of FosGFP labeled neurons (Figure 4e) had a significant main effect of acute injection [$F_{(1,20)} = 7.818$, $p = 0.011$]. LSD adjusted simple main effects showed rats administered repeated amphetamine had significantly more FosGFP expression after 1 mg/kg rather than 0 mg/kg acute amphetamine injection ($p = 0.026$). Though expected based on the previous findings, in Fos-GFP transgenic rats we did not see a difference between repeated drug administration groups acutely injected with 1 mg/kg amphetamine, which may be an effect of the labeling FosGFP in place of Fos, or as has been found before, protein labeling may be different between different strains of rodent (Conversi et al., 2011). It is worth noting that average numbers of Fos-expressing cells in all immunofluorescent findings have been lower than found with DAB immunohistochemistry. Regardless, in line with other

previous findings, we showed, as expected expected, labeling of ΔFosB neurons coincided with repeated drug administration (Figure 5F). A two-way ANOVA (repeated administration x acute injection) analyzing ΔFosB expression had a significant main effect of repeated administration [$F_{(1,20)} = 13.64$, $p = 0.001$], and LSD adjusted simple main effects that revealed significant differences between repeated drug administration group when acutely injected with either 0 mg/kg ($p = 0.018$) or 1 mg/kg amphetamine ($p = 0.016$).

Finally, we looked for co-expression of FosGFP and ΔFosB in Fos-GFP transgenic rats that underwent amphetamine sensitization and found increased recruitment of ΔFosB-expressing neurons to the Fos-expressing neuronal ensemble of rats exhibiting locomotor sensitization (Figure 4G). A two-way ANOVA (repeated administration x acute injection) showed significant main effects of both repeated administration [$F_{(1,20)} = 5.985$, $p = 0.024$] and acute injection [$F_{(1,20)} = 13.54$, $p = 0.002$], with no significant interaction. Bonferroni post-hoc comparisons revealed more FosGFP/ΔFosB colabeled neurons in rats repeatedly administered amphetamine and acutely injected with 1 mg/kg amphetamine when compared to rats repeatedly administered saline ($p = 0.029$) or acutely injected with 0 mg/kg amphetamine ($p = 0.006$). This revealed that neurons activated during repeated amphetamine administration were more likely to also be activated during locomotor sensitization, which may indicate more previously activated neurons are required to encode locomotor sensitization.

Figure 5: Fos/ΔFosB co-expression in the accumbens after locomotor sensitization

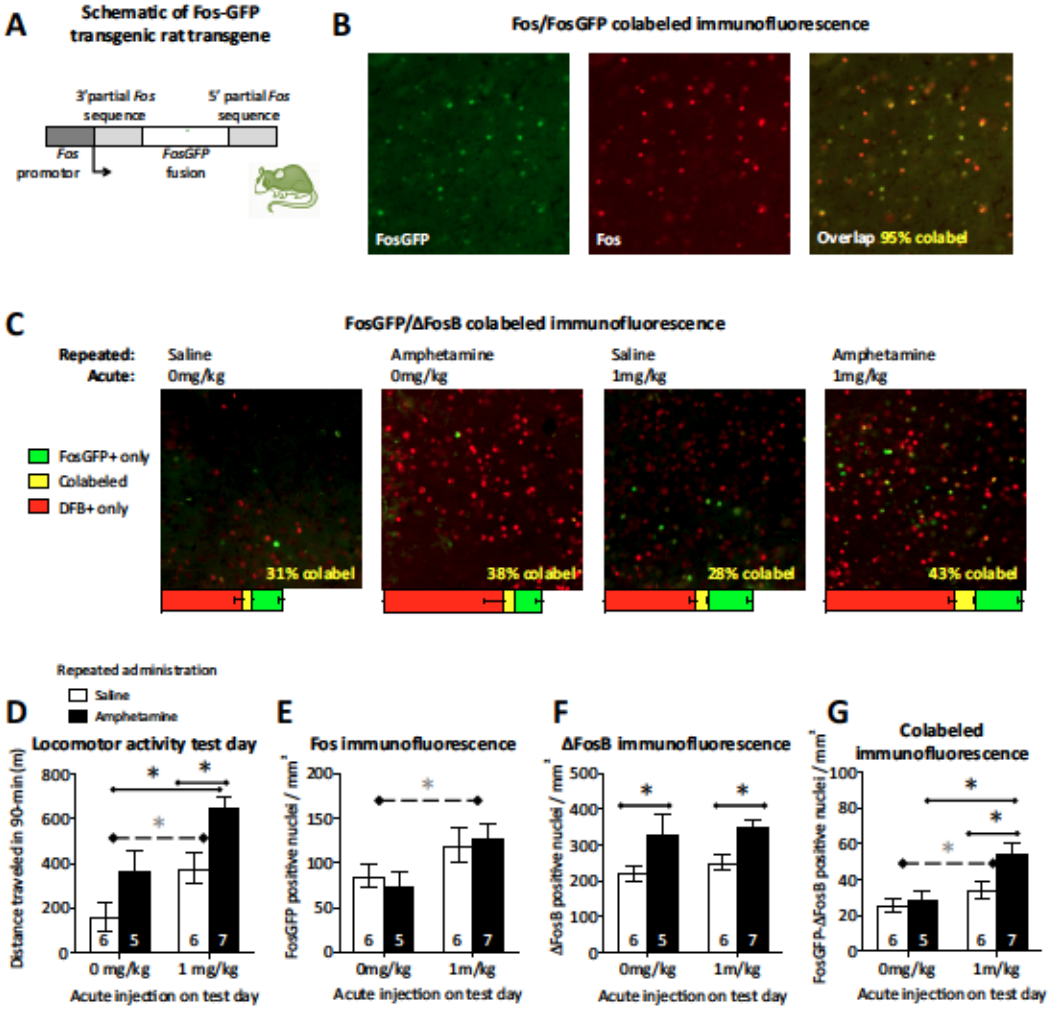


Figure 5.

A) Schematic of Fos-GFP transgenic rat. B) Representative images of FosGFP and Fos expression and co-expression with immunofluorescence. C) Representative images and diagram of FosGFP/ΔFosB co-expression with immunofluorescence (DFB = ΔFosB). D) Locomotor activity of Fos-GFP transgenic rats sacrificed for FosGFP/ΔFosB colabeling. E) Fos immunofluorescence, F) ΔFosB immunofluorescence, and G) Co-expression of FosGFP/ΔFosB in nucleus accumbens shell.

5. Experiment 5: Learned associations influenced locomotor sensitization and Fos-expression

5.1. Learned associations of context-specific locomotor sensitization

In order to determine if locomotor sensitization we observed in rats repeatedly administered amphetamine and acutely injected with 1 mg/kg amphetamine on test day was a result of learned associations to context, we developed controls for our amphetamine sensitization protocol to test for context-specific locomotor sensitization. We conditioned rats with repeated drug administration in a novel context (context B) that was distinct from the testing locomotor chambers (context A) (Figure 6A). Prior to sensitization conditioning in either context, rats were habituated for 2-hours in context A to reduce excessive novelty exploration on test day. Locomotor activity recorded during these habituation sessions prior to repeated drug administration was similarly low for all rats (150.64 ± 0.81 meters).

Ultimately, tests revealed locomotor sensitization was consistent with what we have previously found when rats were conditioned and tested in the same context, but additionally found that conditioning in an alternate context prevented locomotor sensitization. A three-way ANOVA (conditioning context x repeated administration x acute injection) of locomotor activity on test day was not significant. Because we wanted to compare locomotor sensitization by context, we next ran a two-way ANOVAs (repeated administration x acute injection) to compare locomotor activity for each separate conditioning context (Figure 6B). For context A conditioned rats, we found a significant interaction [$F(_{1,51}) = 8.762$, $p = 0.047$] between repeated administration and acute injection, as well as significant main effects of repeated

administration [$F_{(1,51)} = 21.18, p < 0.001$] and acute injection [$F_{(1,51)} = 134.7, p < 0.001$]. As seen previously for context A conditioned rats, Bonferroni post-hoc tests showed significant increases in locomotor activity of amphetamine sensitized rats acutely injected with 1 mg/kg amphetamine when compared to rats administered repeated saline ($p < 0.001$) or acute injection with 0 mg/kg amphetamine ($p < 0.001$). For context B conditioned rats, we found no significant interaction, nor main effect of repeated administration, but did find a significant main effect of acute injection [$F_{(1,40)} = 55.67, p < 0.001$]. LSD simple main effects showed that rats acutely injected with 0 mg/kg or 1 mg/kg amphetamine had significant differences when previously administered repeated amphetamine ($p < 0.001$) or saline ($p < 0.001$). Further, Bonferroni post-hoc analysis confirmed there was no significance between repeated drug administration groups after acute injection with 1 mg/kg amphetamine, meaning there was no significant locomotor sensitization in rats conditioned in a different context from which they were tested.

5.2. Fos expression after context-specific locomotor sensitization

We next considered how the Fos-expressing neurons reflected the differences rats exhibitied in locomotor sensitization when they were conditioned with repeated amphetamine administration in a different context from which they were tested for sensitization. Because histological results of the nucleus accumbens were best represented by effects seen in the accumbens shell, we chose to limit analysis to accumbens shell for all future experiments.

We found rats conditioned in context A had increased Fos expression in the accumbens shell of rats exhibiting locomotor sensitization (Figure 6C, panel 1). A two-

way ANOVA (repeated administration x acute injection) revealed a significant interaction [$F_{(1,51)} = 5.448, p = 0.024$] as well as significant main effects for repeated administration [$F_{(1,51)} = 10.53, p = 0.002$] and acute injection [$F_{(1,51)} = 7.873, p = 0.007$]. Consistent with our previous findings from rats conditioned and tested in the same context, Bonferroni post-hoc tests confirmed Fos expression was significantly increased in amphetamine sensitized rats acutely injected with 1 mg/kg amphetamine when compared to rats administered repeated saline ($p = 0.001$) or acutely injected with 0 mg/kg amphetamine ($p = 0.004$). We further confirmed a correlation between locomotor activity and Fos expression in the accumbens shell ($r = 0.449, p < 0.001$) for rats conditioned in the same context in which they are tested.

We found rats conditioned in context B did not have Fos expression increases in accumbens shell after repeated amphetamine administration and acute injection with 1 mg/kg amphetamine (Figure 6C, panel 2). A two-way ANOVA (repeated administration x acute injection) found no significant interaction, or main effect of acute injection, which indicated that rats conditioned in context B expressed the same amount of Fos expressed in accumbens shell when tested with 0 mg/kg or 1 mg/kg amphetamine in a different context from which they were conditioned. We did, however, find a significant main effect of repeated administration [$F_{(1,61)} = 4.233, p = 0.044$]. Yet, LSD adjusted simple main effects showed no significant differences between repeated drug administration group for either acute injection dose. We found no correlation between the locomotor activity on test day and Fos expression ($r = 0.626$, $p = 0.085$) for rats conditioned in a different context from which they were tested.

Therefore, in the accumbens shell, we revealed a context-specific nature of Fos expression dependent on whether rats were conditioned in the same or an alternate context from which they were tested. We also found Fos expression only correlated to locomotor sensitization when rats were conditioned and tested in the same context. These results confirmed that earlier findings about locomotor sensitization after our amphetamine sensitization protocol did allow for observation of learned associations between context and drug effects that may be reflected in the Fos-expressing neuronal ensemble.

5.3. ΔFosB expression after context-specific locomotor sensitization

Lastly, to confirm that learning in context B for the different repeated drug administration groups was similar to learning in context A, we analyzed ΔFosB expression in the accumbens shell by conditioning context. As previously seen, we found increased ΔFosB expression when rats were previously administered repeated amphetamine (Figure 6D, panel 1). A two-way ANOVA (repeated amphetamine x acute injection) analyzing ΔFosB expression in the accumbens shell of rats conditioned in context A revealed a main effect of repeated administration [$F_{(1,51)} = 38.65$, $p < 0.001$]. LSD adjusted simple main effects showed rats administered repeated amphetamine had significantly increased ΔFosB expression compared to rats repeatedly administered saline whether acutely injected with 0 mg/kg ($p < 0.001$) or 1 mg/kg amphetamine ($p < 0.001$). Bonferroni adjusted post-hoc tests also revealed that there was no difference between rats acutely injected with 0 mg/kg or 1 mg/kg amphetamine after repeatedly administered amphetamine, indicating that sensitization conditioning with repeated amphetamine drove ΔFosB expression, possibly through

learned associations between context and drug effects. ΔFosB expression changed in exactly the same way for rats conditioned in context B (Figure 6D, panel 2). A two-way ANOVA (repeated administration x acute injection) analyzing ΔFosB expression in the accumbens shell of rats conditioned in context B revealed a main effect of repeated administration [$F_{(1,40)} = 38.65$, $p < 0.001$]. LSD adjusted simple main effects showed rats administered repeated amphetamine had significantly increased ΔFosB expression compared to rats repeatedly administered saline whether acutely injected with 0 mg/kg ($p = 0.001$) or 1 mg/kg amphetamine ($p < 0.001$). Bonferroni adjusted post-hoc tests also revealed that there was no difference between rats acutely injected with 0 mg/kg or 1 mg/kg amphetamine after repeatedly administered amphetamine. Therefore, conditioning context did not matter for ΔFosB expression to increase. Instead, only repeated drug administered effected ΔFosB expression. We can infer that similar activity during conditioning meant that rats conditioned in either context were making associations between their environment and the drug rewards, but those associations were only useful to exhibit locomotor sensitization when rats were tested in the same context in which the associations were formed.

6. Experiment 6: Fos/ΔFosB increased co-expression after context-specific locomotor sensitization

Lastly, we wanted to determine how recruitment of the ΔFosB-expressing conditioning ensemble is recruited for context-specific learning. We conditioned Fos-GFP transgenic rats with repeated amphetamine administration in context A, context B, or in the home cage and tested in context A with 1 mg/kg.

First, we measured locomotor activity, and found, as expected, rats conditioned and tested in the same context exhibited sensitized locomotor activity (Figure 6E, black bars). A one-way ANOVA (context) was significant [$F_{(2,14)} = 4.391$, $p = 0.033$]. LSD adjusted comparisons showed rats had increased locomotor activity when tested in the same context A, as compared to rats conditioned in context B ($p = 0.016$), and home cage ($p = 0.054$), but that rats sensitized to amphetamine in context B had the same locomotor activity as rats sensitized to amphetamine in the home cage.

Next, we labeled for immunofluorescence of FosGFP and ΔFosB to ultimately determine how co-expression of these neurons is affected by contextual learning. As found previously with our Fos-GFP transgenic rats, labeled for FosGFP, there was no significant increase in FosGFP expression found between the 3 contexts, despite differences in locomtor activity, shown by one-way ANOVA (Figure 6E, green and yellow bars combined). It is still unclear whether this peculiar result is a result of labeling procedures, strain of rat, or some other factor. We did, however, successfully find increased ΔFosB expression that matches previous labeling. Rats conditioned in either of the novel contexts had increased ΔFosB expression, while home cage conditioned rats had lower ΔFosB expression (Figure 6E, red and yellow bars combined). A one-way ANOVA (context) revealed a significant effect of context on ΔFosB labeling between across groups [$F_{(2,14)} = 8.714$, $p = 0.003$]. LSD adjusted comparisons for rats conditioned in context A or context B were insignificant, as expected; however, rats sensitized to amphetamine in the home cage had significantly less ΔFosB expression than rats conditioned in context A ($p = 0.001$) and context B ($p = 0.008$). The lower baseline ΔFosB expression when conditioned in a familiar context

is a unique finding that may support the idea that ΔFosB is labeling neurons activated during conditioning to encode learned associations between conditioning context and amphetamine.

Finally, colabeling of FosGFP and ΔFosB in the accumbens shell after locomotor sensitization across contexts was significantly increased in rats exhibiting locomotor sensitization, as revealed by one-way ANOVA (context) [$F_{(2,14)} = 5.974$, $p = 0.013$] (Figure 6E, yellow bars). LSD adjusted comparisons revealed rats that showed locomotor sensitization after they were conditioned and tested in context A, and also showed significantly increased co-expression of FosGFP and ΔFosB as compared to rats conditioned in context B ($p = 0.031$) or the home cage ($p = 0.006$), while rats conditioned in context B had no differences in co-expression when compared to rats conditioned in the home cage. These unique results indicated ΔFosB-expressing conditioning ensembles are recruited to the Fos-expressing neuronal ensemble in a context-specific manner, that may be dependent on learned associations that are used to encode locomotor sensitization to amphetamine.

Figure 6: Establishing a behavior model for Fos-inducing context-specific locomotor sensitization

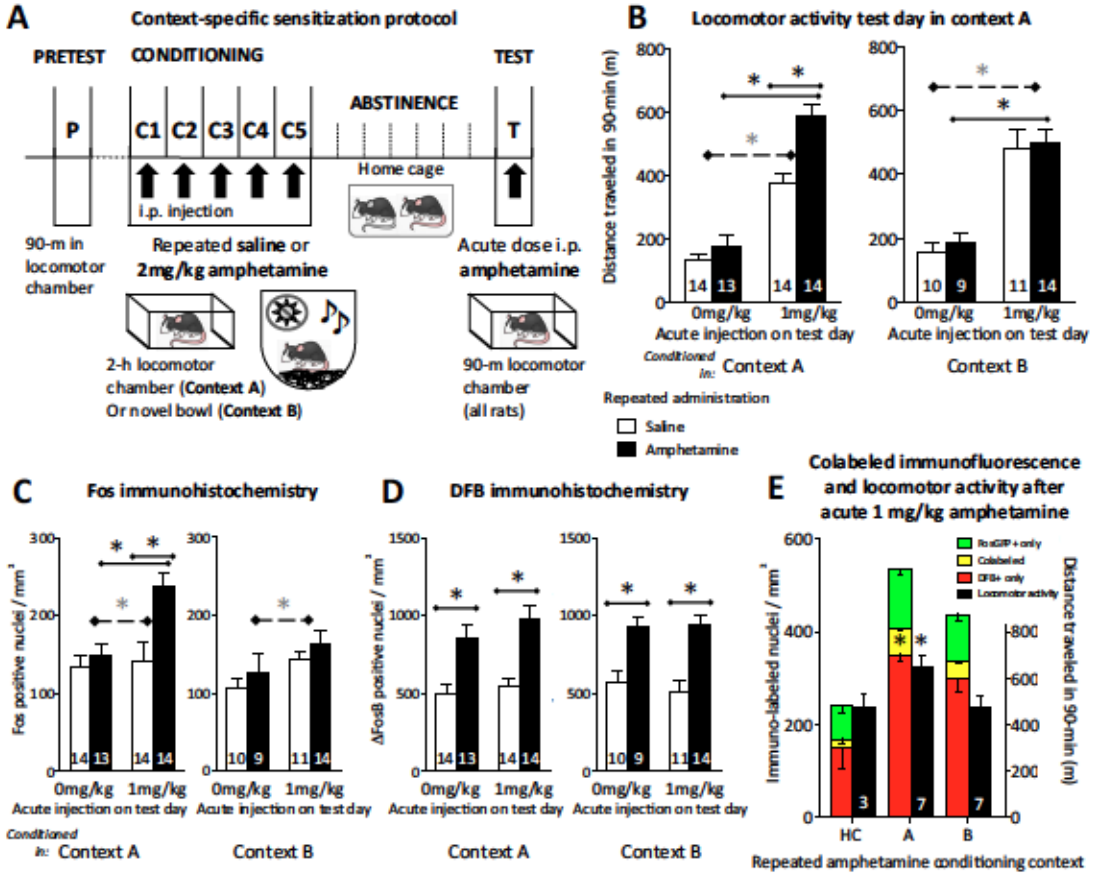


Figure 6.

A) Schematic of context-specific locomotor sensitization protocol. B) Locomotor activity expressed after amphetamine sensitization in context A or conext B sacrificed for immunohistochemistry. C) Fos immunohistochemistry or D) ΔFosB of rats tested for context-specific locomotor activity. E) Locomotor activity (back bars) and immunofluorescence labeling of FosGFP (green and yellow), ΔFosB (red and yellow), and co-expression of FosGFP/ΔFosB (yellow) in rats sensitized to amphetamine in various contexts after injection with 1 mg/kg of amphetamine.

7. Experiment 7: Fos-expressing neuronal ensembles played a role in context-specific locomotor sensitization

Here, we utilized the Daun02 inactivation procedure to ablate neurons which were Fos-expressing after context-specific amphetamine sensitization. This experiment allowed us to test for a causal role of the increased Fos-expressing neurons found after amphetamine locomotor sensitization. We limited experiments only to rats acutely injected with 1 mg/kg amphetamine on test day, and compared how Daun02 or vehicle microinjections into the nucleus accumbens shell of Fos-LacZ rats differentially effects locomotor activity when rats were repeatedly administered amphetamine or saline in context A or context B (Figure 7A).

To induce Fos expression for Daun02 inactivation, we injected all rats with 1 mg/kg amphetamine and measured locomotor activity in context A on induction day. Rats repeatedly administered amphetamine in context A showed sensitized locomotor activity on induction day, while rats conditioned in context B did not (Figure 7B). A two-way ANOVA (conditioning context x repeated administration) showed no significant interaction, or effect of context, but did show a significant main effect of repeated administration [$F_{(1,48)} = 6.45$, $p = 0.014$]. LSD adjusted simple main effects between administration drug groups revealed significantly sensitized locomotor activity when conditioned in context A ($p = 0.010$) and no difference between repeated drug administration groups when conditioned in context B. This aligned with previous findings for context-specific locomotor sensitization, and indicated that learned associations to the conditioning context and drug effects are influencing locomotor sensitization in rats.

Immediately following the induction day session, Fos-LacZ transgenic rats were microinjected with Daun02 or vehicle in the accumbens shell. A few days later we tested all rats with a 1 mg/kg acute amphetamine injection and recorded locomotor activity for changes after Daun02 inactivation. Ultimately, we found rats repeatedly administered amphetamine in context A decreased locomotor activity after Daun02 microinjection, while rats conditioned in context B showed opposite effects (Figure 7C). A three-way ANOVA (conditioning context x repeated administration x microinjection) yielded no significant interaction. To best determine the effect Daun02 was having on learned associations driving context-specific locomotor sensitization, we next ran a two-way ANOVA (conditioning context x microinjection) splitting analysis by repeated drug administration group (note: graphs were split by context, while analysis was split by repeated administration group). We found a significant interaction for rats repeatedly administered amphetamine [$F_{(1,29)} = 4.948$, $p = 0.034$], with no significant main effects or significant Bonferroni post-hoc comparisons. For rats repeatedly administered saline, there was no two-way interaction between conditioning context and microinjection; however, there was a main effect of context on test day locomotor activity [$F_{(1,15)} = 8.604$, $p = 0.010$]. LSD adjusted simple main effects show Daun02 microinjections had a significant effect decreasing locomotor activity in rats conditioned with saline in context A ($p = 0.016$), but not context B ($p = 0.651$). It is unclear why saline conditioned rats injected with Daun02 would decrease locomotor activity on subsequent testing and may be a product of small test groups.

Analysis of β-galactosidase labeling with X-gal was done for each group to determine how effective Daun02 was at ablating neurons that were Fos-expressing. We

found all groups injected with Daun02 had generally decreased levels of β-galactosidase expression regardless of context (Figure 7D). A two-way ANOVA (conditioning context x microinjection) splitting analysis by repeated drug administration group, revealed no significant interactions, but did yield significant main effects of microinjection for rats repeatedly administered either amphetamine [$F_{(1,29)}$ = 19.7, $p < 0.001$] or saline [$F_{(1,15)}$ = 5.457, $p = 0.034$], regardless of context. However, LSD adjusted main effects found between vehicle and Daun02 microinjections were significant for amphetamine rats conditioned in context A ($p < 0.001$) (Figure 7E), but not context B, and there were no significant main effects of context for rats repeatedly administered saline. This tells us that although decrease in β-galactosidase staining was seen in all conditioning and repeated administration groups injected with Daun02, there was a more significant loss of Fos-expressing, β-galactosidase positive neurons in rats that showed context-specific locomotor sensitization on Fos-induction day than other groups.

To take it one step further, we compared within-subject contributions of the Fos-expressing neuronal ensemble that was ablated on Fos-induction day by comparing locomotor activity recorded pre- (Fos-induction day) and post- (test day) microinjection (Figure 7F). A multifactorial, repeated measure ANOVA (pre/post x conditioning context x repeated administration x drug microinjected) yielded no significant interaction. However, it did reveal a significant three-way interaction of locomotor activity before and after microinjection (pre/post x conditioning context x microinjection) [$F_{(1,44)}$ = 5.026, $p = 0.027$]. Sidak post-hoc comparisons revealed significant decreases in locomotor activity from Fos-induction day to test day after

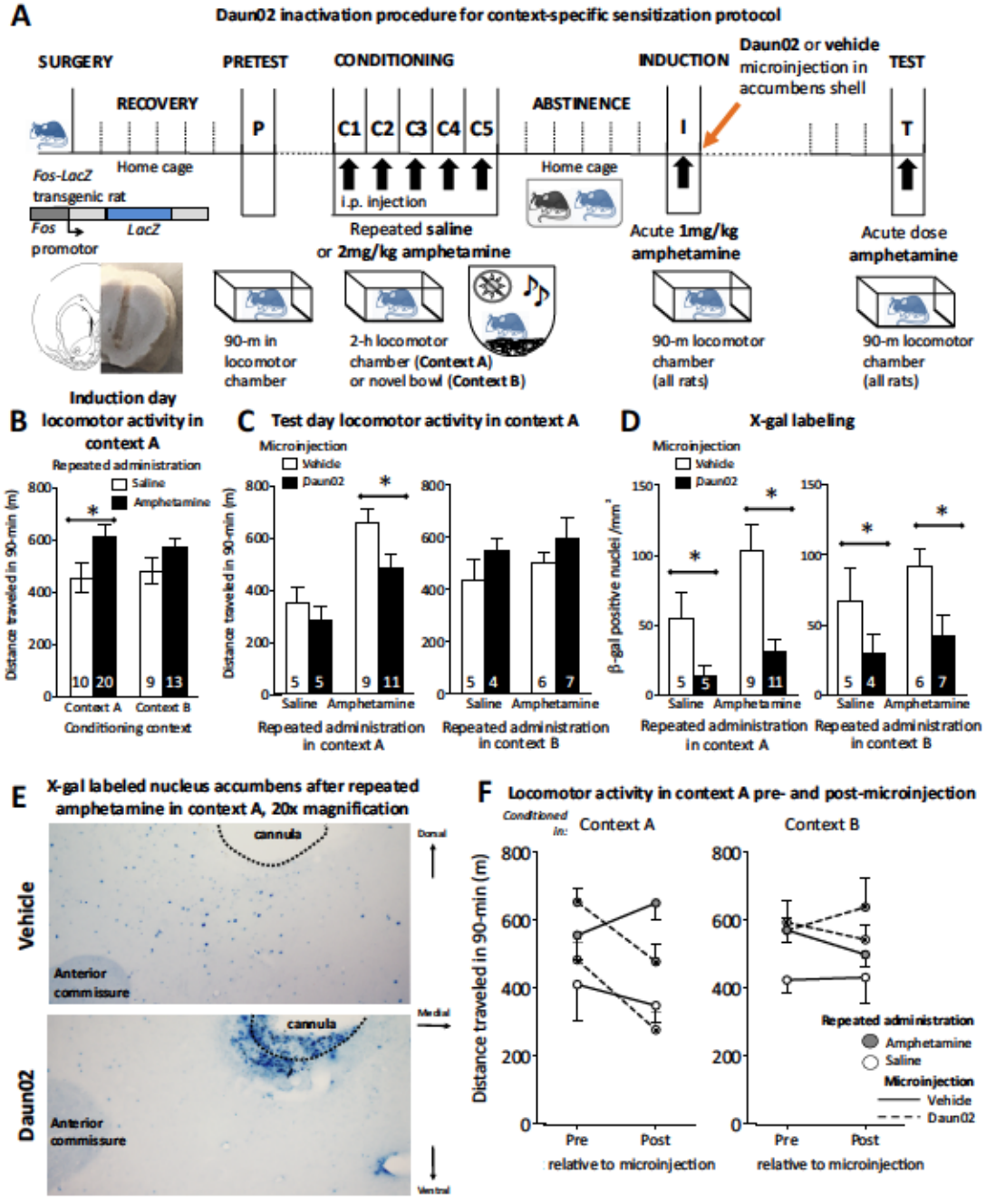
Daun02 microinjection in rats conditioned in context A for both repeated drug administration groups (amphetamine, $p = 0.003$; saline, $p = 0.017$).

In summary, ablation of Fos-expressing neurons in the accumbens shell with Daun02 inactivation after repeated drug administration in context A decreased locomotor activity, and disrupted context-specific locomotor sensitization, while rats conditioned in context B had no change in locomotor activity after Daun02 microinjections. Rats injected with Daun02 in all conditioning groups had a decreased β-galactosidase expression and therefore fewer activated neurons on test day. However, the loss of these neurons was only functionally relevant in rats conditioned in context A. This shows a causal role of Fos-expressing neurons in the accumbens shell of amphetamine sensistized rats encode context-specific information that drive locomotor sensitization.

Figure 7. (on following page)

A) Schematic of Daun02 induction for amphetamine sensitization. B) Locomotor activity on induction day. C) Locomotor activity and D) X-gal staining on test day, after microinjection to the accumbens shell with Daun02 or vehicle. E) Representative images of nucleus accumbens after vehicle or Daun02 injection. F) Comparison of rat behavior pre- and post- microinjection.

Figure 7: Establishing causal role of Fos-expressing neurons in context-specific sensitization



Chapter 3: Discussion

1. Conclusions

Our findings on behavioral sensitization and related nucleus accumbens Fos expression were consistent with others in the literature (Robinson and Becker, 1986; Uslaner et al., 2001; Mattson et al., 2007a). However, we were able to provide a more in-depth characterization of the Fos-expressing neuronal ensemble, and its role in learned associations of locomotor sensitization. Ultimately, these experiments supported our hypothesis that nucleus accumbens Fos-expressing neuronal ensembles encoding context-specific sensitization played a causal role in amphetamine induced locomotor activity.

1.1. Characterization of Fos-expressing neuronal ensembles encoding locomotor sensitization

Similar to previous studies (Robinson and Becker, 1986; Mattson et al., 2007b), we confirmed low, intermittent doses of repeated amphetamine can induce sensitized locomotor activity in rats by showing reliable amphetamine locomotor sensitization after 5-days repeated amphetamine (2 mg/kg i.p.) administration, 1-week abstinence, and acute 1 mg/kg amphetamine injection without inducing restrictive stereotypies. Additionally, increased Fos expression and ΔFosB expression in the nucleus accumbens shell and core supported previous findings about locomotor sensitization and immediate early gene expression (Chen et al., 1997; Uslaner et al., 2001).

While Fos expression in the nucleus accumbens was typically increased after locomotor sensitization, ΔFosB changes were not related to behavioral sensitization recorded test day after acute amphetamine injections. Instead, ΔFosB labeled neurons

active during sensitization conditioning. Previous studies have found similar increases in ΔFosB expression after a variety of sensitizing conditions (Chen et al., 1997; Beloate et al., 2016a; Carneiro de Oliveira et al., 2016) with similar dissociation between ΔFosB expression and behavioral sensitization exhibited test day (Beloate et al., 2016b). Additionally, for the first time, we observed the overlap of the Fos-expressing neuronal ensemble and ΔFosB-expressing conditioning ensemble, and found that locomotor sensitization to acute amphetamine injection utilizes more neurons activated during repeated amphetamine administration.

We also added to a conflicting literature about the activation of the accumbens shell and core neurons in addiction-related behaviors, and the involvement of dopamine-receptor subtypes in locomotor sensitization to amphetamine. We found a strong relationship between increased Fos expression of the accumbens shell and locomotor sensitization in rats, and were able to show the accumbens core and shell have similar, proportional activity-dependent changes to Fos and ΔFosB expression after our amphetamine sensitization protocol. Furthermore, RNAscope labeling revealed D1-receptor-expressing neurons were preferentially recruited to nucleus accumbens Fos-expressing ensembles encoding locomotor sensitization, which may help encode sensitization behavior (Kai et al., 2015).

1.2. Evaluation of the role of Fos-expressing neuronal ensembles play in context-specific locomotor sensitization

Context-specific behavioral sensitization studies, with similar characteristics to our protocol for locomotor sensitization, have been done before for a range of stimulants (Uslaner et al., 2001). Here, we tested locomotor sensitization to

amphetamine that relied on learned-associations to environmental cues by conditioning rats with repeated amphetamine administration in different contexts. We revealed changes in expression of sensitized locomotor activity and Fos expression were only significant when rats were repeatedly administered amphetamine and acutely injected with amphetamine in the same contexts. In addition, we found context-specific effects of Fos expression in nucleus accumbens shell on test day that reflected locomotor sensitization. ΔFosB expression was increased after repeated amphetamine administration equally for two different novel contexts, and less for rats conditioned with repeated amphetamine in the home cage, which further suggested ΔFosB expression was occurring as novel learned associations that encode locomotor sensitization are forming. We found changing the context in which rats were conditioned and tested reduced overlap of Fos-expressing neuronal ensembles and ΔFosB-expressing conditioning ensembles.

Findings from ablation of Fos-expressing neurons in the accumbens shell after an induction test and Daun02 inactivation established a causal role of the neuronal ensemble in locomotor sensitization. We saw effects only after deletion of Fos-expressing neuronal ensembles in rats sensitized in a context specific manner, despite decreased Fos-expression, as labeled by β-galactosidase, across all rats microinjected with Daun02. Other studies have also shown a causal role for the small population of Fos-expressing neuronal ensembles in the nucleus accumbens after cocaine behavioral sensitization (Koya et al., 2016) and other addiction-related behaviors relying on learned associations (Fanous et al., 2012; Cruz et al., 2014; Caprioli et al., 2017).

Our findings about the causal role of Fos-expressing neuronal ensembles in context-specific locomotor sensitization, and the variations we found in recruitment of neurons encoding learned associations to context-specific locomotor sensitization add to a rich, trending literature about the importance of neuronal ensembles.

2. Future directions

As we have generally identified a behavioral model to study conditioned learning, there are many new questions that can be answered about the Fos-expressing neuronal ensemble. Though not a comprehensive list, three major lines of investigation to consider would be (i) the comparison between globally found neuronal alterations and unique alterations in Fos-expressing neurons, (ii) the testing of established, relevant neuronal subtypes in an activity-dependent manner, and (iii) the manipulation of the sensitization protocol to alter encoding of learned associations in context-specific locomotor sensitization. Here, I will elaborate on these directions, indicating some research already exploring these areas, and other interesting targets for consideration.

Using the context-specific amphetamine sensitization protocol, examination of known global neuronal alterations could be measured in the behavior encoding Fos-expressing neuronal ensemble. Established methods to determine molecular, cellular, and electrophysiological properties of the cell could be combined with transgenic or viral technologies to measure unique alterations in strongly-activated neurons. In fact, experiments using FACS (Liu et al., 2014; Li et al., 2015; Rubio et al., 2016), analyzing dendritic spine morphology (Grueter et al., 2013; Singer et al., 2016), and recording with slice electrophysiology (Koya et al., 2012; Whitaker et al., 2017) have identified a handful of potential mechanisms for encoding learning. These studies tended to used

transgenic rodent models or targeted brain infection with viral vectors encoding designer genes behind a *Fos*-promoter, or another immediate early gene promoter. However, it is possible with non-transgenic labeling techniques to identify these neurons for cellular changes (Liu et al., 2014; Singer et al., 2016). Additional interesting learned-association targets that are known to induce global neural adaptations after amphetamine sensitization include surface receptors (AMPA: (Wolf, 2016; Wang et al., 2017a); NMDA: (Li et al., 2016; Voyer et al., 2017) , μ-opioid: (Kuo et al., 2016)), proteins modulating receptor expression (arrestin: (Zurkovsky et al., 2017); cadherin: (King et al., 2017)), regulatory peptides (ghrelin: (Jang et al., 2017), Cdk5/p35: (Mlewski et al., 2016)) and roles of long non-coding RNAs(Zhu et al., 2015). Therefore, comparing changes in these molecular targets for Fos-expressing and non-Fos-expressing neurons after amphetamine locomotor sensitization could prove fruitful for determining mechanisms encoding conditioned learning.

Another interesting line of research would be testing the role of neuron subtypes recruited to the Fos-expressing population. Here, we showed a heterogeneous subtype of accumbens neurons that are both *D1*-, *D2*-, and non-dopamine-receptor expressing were recruited to the *Fos*-expressing neuronal ensemble that plays a role in learned associations of amphetamine sensitization. Manipulating the enhanced D1-receptor population that we found were recruited to encode sensitized locomotor activity may elucidate circuitry needed for conditioned learning. For example, rats with transgenes encoding the bacterial protein cre-recombinase under a *D1-receptor* promoter could be combined with a viral vector, that carries a cre-dependent gene, that would selectively manipulate neurons co-expressing D1-receptors and Fos. Additionally, testing

specifically activated neurons from other neural subtypes found in nucleus accumbens, such as parvalbumin expressing interneurons found to be required for amphetamine sensitization when globally inhibited (Wang et al., 2017b), may introduce a critical sub-population within the Fos-expressing neuronal ensembles driving learned behavior.

Also of potential interest is the ΔFosB-expressing conditioning ensemble our study found that labels activity during sensitization conditioning. Further exploration of the role ΔFosB directly plays, or the role neurons ΔFosB labels play, after locomotor sensitization expression merits investigation. In other studies, ΔFosB overexpression has been linked to differential modification of the synaptic properties of medium spiny neurons for D1-receptor- and D2-receptor-expressing neurons, as well as different modifications by accumbens sub-areas (Grueter et al., 2013). Future experiments could test synaptic properties of Fos/ΔFosB co-expressing neurons, additionally evaluating dopamine-receptor subtypes of medium spiny neurons or differences between accumbens shell and core, and could possibly yield new mechanisms of learning.

Lastly, manipulation of the amphetamine sensitization protocol may highlight different dynamics of the Fos-expressing neuronal ensemble. One manipulation could be to extend the withdrawal period. Recent studies altering withdrawal periods between context-specific sensitization conditioning and behavioral sensitization testing found the longer memories are incubated, the more generalized learned associations become and context-specificity is lost (Engelke et al., 2017). Testing our protocol with varied withdrawal periods may expose a transition of neuronal ensembles to other brain areas, or a refinement or expansion of the Fos-expressing ensemble after incubation. The role of Fos-expressing neuronal ensembles in incubation of operant learning have already

begun to be tested for forced abstinence (Fanous et al., 2012) and voluntary abstinence (Caprioli et al., 2017). Other interesting protocol manipulations could be to house rats in an enriched environment before sensitization. This exposure has already shown to decrease novelty related amphetamine sensitization (Garcia et al., 2017), but may change how Fos expression in the nucleus accumbens correlates with locomotor sensitization behavior. Since our experiments found novelty-dependent effects of ΔFosB expression, as rats conditioned in the novel contexts had more ΔFosB expression than rats conditioned in the home cage, it would be particularly interesting to see how Fos/ΔFosB co-expression changes after context-specific amphetamine locomotor sensitization when rats are housed in enriched environments.

As with many experiments, our results created more questions than we can answer here. With better technologies and methods in place, studying neuronal ensembles of highly activated neurons is becoming more available, and should be considered for studying addiction-related learning in the future.

3. Summary

Studying a small, activity-dependent population of neuronal ensembles has proven, with previous studies, to be useful in identifying unique alterations that are distinct from global changes in the same areas, particularly for addiction-related behaviors. Here, we established a causal role that Fos-expressing neuronal ensembles played in the nucleus accumbens to encode context-specific locomotor sensitization to amphetamine. We characterized distinct differences in recruitment of dopamine-receptor expressing nucleus accumbens neurons, as well as recruitment of previously activated ΔFosB-expressing neurons. With these experiments, we identified targets for

investigating encoding of learned associations that drive addiction-related behavior. Using our model of learned associations, it is possible to test for unique alterations encoding highly complex learned associations, which can be used to inform development of therapeutics and benefit humans with other brain diseases that affect learning.

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References

Appendix A: Statistical assumptions of data

When comparing differences between groups using ANOVAs, there are three main assumptions that must be met; data must prove (i) independence of cases, (ii) normal distributions of the dependent variable, and (iii) equality of dependent variable variances. For experimental data sets where we ran an ANOVA, we assessed validity of these three assumptions using IBM SPSS 23 and Laerd Statistics guides (Cite Laerd). To satisfy each assumption, we tested for (i) data outliers, (ii) normality, and (iii) homogeneity. Certain analyses also involved extra tests for linearity, sphericity, etc. as explained below.

Independence of cases via outliers was typically identified graphically by boxplot for data points 1.5 (outliers) or 3.0 box-lengths (extreme outliers) from the edge of the boxplot. In some instances, we used assessment of studentized residuals (quotient resulting from division of a residual by the estimation of its standard deviation) for absolute values greater than 3. Values greater than 3 were further assessed, while all lesser numbers were accepted in the data set.

To test for normality of data, we used the Shapiro-Wilk's test and identified values that were greater than 0.05 as compliant with normality. Within the various ANOVAs, if a single group (defined by the collection of factors for the ANOVA being tested) did not meet this criteria for normality, assessment of the Normal Q-Q plot was performed to examine general linearity of the data. For Normal Q-Q plots deemed non-linear, we reassessed and accepted Shapiro-Wilk's test values greater than 0.05 divided by the number of Shapiro-Wilk's tests being run (number of groups).

To test for homogeneity of variance, we used a Levene's test for equality of error variance accepting data sets with values greater than 0.05. Where noted, we also used a Box's M test for homogeneity of covariance matrices. We proceeded with analysis of data sets that violated homogeneity of repeated measure testing when the variance ratio between the group with the largest and smallest variance were less than 3.

Under these analyses, most data sets were compliant with assumptions. Any occasion where assumptions were not met is noted in the results. However, for all data sets reported, we proceeded with the statistical analysis, despite any failed assumptions, based on the robustness of the ANOVAs being performed, consistency in group sizes within data sets, and to avoid transformations of data that would prevent other statistical groups from meeting test criteria.

For mixed model ANOVAs and repeated measure ANOVAs, we also determined preferred analysis metrics based on Mauchly's test of sphericity. If the sphericity test was violated ($p < 0.05$) we examined the epsilon (ϵ) values of the data. In all cases where sphericity failed, we chose to accept the Greenhouse-Geiser analysis, reporting original df-values.

A.1. Experiment 1: Confirming amphetamine sensitization protocols elicit sensitization conditioning

A.1.1. Repeated drug administration effects on conditioning assessed by repeated measure two-way ANOVA

There were three outliers, as assessed by inspection of a boxplot. However, there were no outliers, as assessed by further examination of the absolute value of studentized

residuals for values greater than 3. Data was distributed normally ($p > 0.05$) with the exception of saline rats on sessions 4 and 5 ($p = 0.002$ and 0.015, respectively), as assessed by Shapiro-Wilk's test. However, Normal Q-Q plots were visual assessment for these groups and deemed linear. There was not homogeneity of variances on any repeated administration session, as assessed by Levene's test of homogeneity of variance ($p < 0.05$). Nor was there homogeneity of covariances, as assessed by Box's test of equality of covariant matrices ($p < 0.001$). We then calculated the variance ratio of the group with the highest variance to the lowest variance, which was less than 3. Mauchly's test of sphericity indicated that the assumption of sphericity was violated for the two-way interaction, χ-square = (50.265), $p < 0.001$). We therefore reported Greenhouse-Geisser analysis ($\epsilon = 0.600$).

A.1.2. Repeated drug administration effects on locomotor activity with dose dependent testing assessed by two-way ANOVA

There were two outlier rats in the data set, as assessed in a boxplot. Further analysis of studentized data yielded no values greater than the absolute value of 3. Data were normally distributed ($p > 0.05$), with the exception of rats repeatedly administered amphetamine and acutely administered 0 mg/kg ($p = 0.011$) and 2 mg/kg amphetamine ($p = 0.49$), as assessed by Shapiro-Wilk's test. Normal Q-Q plots were assessed visually for these groups and deemed linear. The assumption of homogeneity of variances was violated as assessed by Levene's test for equality of variances ($p = 0.041$). We chose to continue with analysis, despite this violation due to the robustness of the ANOVA.

A.1.3. Repeated drug administration effects on binned-locomotor activity with dose dependent testing assessed by three-way mixed model ANOVA

There were few data outliers in individual bins, as assessed by inspection of a boxplot. We chose to leave these unique data points as a part of the data set to be analyzed, as no individual rat had an excessive number of bins in which they were outliers. The binned data was not normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$); however, they were normally distributed, as visually assessed by Normal Q-Q Plot. There was homogeneity of variances in most bins, as assessed by Levene's test for equality of variances ($p > 0.05$). Concern about non-homogeneity of variance in the few bins without homogeneity was disregarded because of approximately equal sample size per treatment group, and the robustness of the three-way mixed ANOVA.

Mauchly's test of sphericity indicated that the assumption of sphericity had been violated [χ-square(152) = 306.095, $p < 0.001$]. We therefore reported Greenhouse-Geisser analysis ($\epsilon = 0.469$).

A.1.4. Repeated drug administration effects on binned-stereotypic behavior with dose dependent testing assessed by three-way mixed model ANOVA

We confirmed data had normal Gaussian distributions by histogram, and proceeded with parametric testing.

A.2. Experiment 2: Establishing existence of a Fos-expressing neuronal ensemble in the nucleus accumbens

A.2.1. Locomotor activity of rats sacrificed for immunohistochemistry by two-way ANOVA

Residual analysis was performed to test for the assumptions of the two-way ANOVA. There were no outliers, as assessed by inspection of a boxplot. Data was normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$), and confirmed with Normal Q-Q plot. The assumption of homogeneity of variances was violated, as assessed by Levene's test for equality of variances ($p = 0.003$). We chose to carry on regardless due to equal group size.

A.2.2. Combined accumbens areas Fos expression assessed by two-way M(ultiway)ANOVA, and two-way ANOVAs by accumbens shell and core separately

First, we assessed the data to ensure assumptions for a 2-way MANOVA were met. There was a linear relationship between the dependent variables (Fos expression in the accumbens shell and Fos expression in the accumbens core), as assessed by scatterplot. There was no evidence of multicollinearity, as assessed by Pearson correlation ($|r| < 0.9$). There were no univariate outliers in the data, as assessed by inspection of a boxplot. There were no multivariate outliers in the data, as assessed by Mahalanobis distance ($p > 0.001$). Number of Fos cells counted in shell and core were normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). There was homogeneity of covariance matrices, as assessed by Box's M test ($p = 0.71$). As these tests were valid for combined data, they were also valid for individual accumbens sub-regions. Data collected for mid-posterior shell and core were not assessed for assumptions.

A.3. Experiment 3: Using RNAscope to determine distribution of dopamine-receptor subtypes within the Fos-expressing neuronal ensemble

A.3.1. Distribution of D1-receptor and D2-receptor expressing neurons between accumbens shell and core by two-way repeated measures ANOVA

We found there were no outliers, as assessed by examination of studentized residuals, for absolute values greater than 3. Studentized residuals of cell quantifications were normally distributed ($p > 0.05$), with the exception of quantification of D1 neurons in the core ($p = 0.37$), as assessed by Shapiro-Wilk's test of normality. However, visual assessment by Normal Q-Q plot was deemed linear. The epsilon derived from Greenhouse-Geisser analysis indicates perfect sphericity (epsilon = 1.00) and therefore we assumed spherecity for interpretation of results.

A.3.2. Distribution of Fos positive nuclei co-expressing dopamine-receptor subtypes in accumbens core by two-way repeated measure ANOVA

There were no outliers in the data, as assessed by inspection of a boxplot. Data was normally distributed for all groups ($p > 0.05$) with the exception of non-dopamine expressing neurons of the shell colabeled for *Fos* in rats repeatedly administered saline ($p = 0.003$), as assessed by Shapiro-Wilk's test. Further analysis of this group by visual inspection of a Normal Q-Q plot was deemed linear. There was homogeneity of variances, as assessed by Levene's test for equality of variances ($p > 0.05$) for all brain area cell types except *D1-receptor*-expressing neurons that were *Fos* positive in the shell ($p = 0.046$). However, the ratio between the largest and smallest variance group was less than 3, and thus compliant. Mauchly's test of sphericity indicated that the assumption of sphericity was violated (χ-square(2) = 8.271, $p = 0.016$). We proceeded with Greenhouse-Geisser analysis ($\epsilon = 0.591$).

A.4. Experiment 4: Determining a relationship between neurons activated during sensitization conditioning and the Fos-expressing neuronal ensembles found after amphetamine-induced locomotor sensitization

A.4.1. Locomotor activity of rats sacrificed for immunohistochemistry by two-way ANOVA

Residual analysis was performed to test for the assumptions of the two-way ANOVA. There were no outliers of the data by inspection of a boxplot. Data from all groups were considered normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$), and confirmed with Normal Q-Q plots. The assumption of homogeneity of variances was violated, as assessed by Levene's test for equality of variance ($p = 0.001$). We chose to carry on regardless of this violation due to group size.

A.4.2. Combined accumbens area Fos expression assessed by two-way MANOVA, and two-way ANOVAs by accumbens shell and core separately

First, we assessed the data to ensure assumptions for a 2-way MANOVA were met. There was a linear relationship between the dependent variables (repeated administration and acute injection), as assessed by scatterplot. There was no evidence of multicollinearity, as assessed by Pearson correlation ($|r| < 0.9$). There were no univariate outliers in the data, as assessed by inspection of a boxplot. There were no multivariate outliers in the data, as assessed by Mahalanobis distance ($p > 0.001$). Number of DFB cells counted in shell were normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). Quantification of DFB cells counted in the core were normally distributed ($p > 0.05$), except in the repeated amphetamine group given 0mg/kg acute amphetamine injection on test day ($p = 0.037$). Analysis of normal

distribution was found compliant after visual inspection of Normal Q-Q plots were deemed linear. There was homogeneity of covariance matrices, as assessed by Box's M test ($p = 0.521$). As these tests were valid for combined data, they were also valid for individual accumbens sub-regions.

A.4.6. Co-expression of FosGFP and ΔFosB in nucleus accumbens shell after locomotor sensitization by three-way ANOVA

We analyzed the residuals of the data to test the assumptions of the two-way ANOVA. There were two outliers assessed by boxplot; however, further analyses of the absolute values for the studentized residuals of these rats were less than 3. All data was normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). There was homogeneity of variances, as assessed by Levene's test for equality of variances ($p = 0.761$).

A.5. Experiment 5: Determining how learned associations to context influence locomotor sensitization and neuronal adaptations

A.5.1. Locomotor activity of context-specific locomotor sensitization for immunohistochemistry by three-way ANOVA

There were four outliers, as assessed by boxplot. The absolute value of studentized residual analysis for these rats were less than 3, with the exception of the single rat conditioned in context B with saline whose residual was 3.27. We chose to leave this rat for the remainder of the analysis due to the robustness of the ANOVA and similar group numbers. All test for normality were valid ($p > 0.05$) as assessed by Shapiro-Wilk's test of normality. However, there was not homogeneity of data as

assessed by Levene's test for equality of variances ($p = 0.042$). We chose to continue with analysis despite lack of homogeneity, due to the robustness of the ANOVA.

A.5.2. Fos expression in nucleus accumbens shell after context-specific locomotor sensitization by three-way ANOVA

There was a single outlier, as assessed by boxplot. The absolute value of the studentized residual for this rat was 3.05, which is greater than 3. We chose to leave this outlier in the data set, due to the robustness of the ANOVA and similar group numbers. Normality was considered valid ($p > 0.05$) for all groups except for rats injected with repeated amphetamine and 0 mg/kg acute amphetamine in either context. For these groups, Normal Q-Q plots were assessed as linear. For all groups homogeneity was found, as assessed by Levene's test for equality of variances ($p > 0.05$).

A.5.3. Fos expression in nucleus accumbens after context-specific locomotor sensitization by three-way ANOVA

There were five outliers, as assessec by boxplot; however, analysis of studentized residuals for these rats were all less than 3. All data sets were found to have normal distribution ($p > 0.05$), as assessed by Shapiro-Wilk's test. There was homogeneity of variances, as assessed by Levene's test for equality of variances ($p > 0.05$).

A.6. Experiment 7: Proving a causal role of Fos-expressing neuronal ensembles in context-specific locomotor sensitization in Fos-LacZ transgenic rats

A.6.1. Context-specific locomotor activity on test day post-Daun02 inactivation by three-way ANOVA

There were 4 outliers, as assessed by boxplot. The absolute values of studentized residuals of these rats were found to be less than 3, with the exception of one rat conditioned in context A with amphetamine and microinjected with Daun02 whose residual was 3.13. We chose to leave this rat for the remainder of the analysis due to the robustness of the ANOVA. Data was normally distributed ($p > 0.05$) with the exception of the context A, amphetamine conditioned group injected with Daun02 ($p = 0.005$), as assessed by Shapiro-Wilk's test. Normal Q-Q plot assessment of this group was deemed linear. There was homogeneity of variances, as assessed by Levene's test for equality of variances ($p = 0.732$).

A.6.2. β-galactosidase expression after Daun02 inactivation by three-way ANOVA

There were 5 outliers, as assessed by boxplot. The absolute values of studentized residuals of these rats was found to be less than 3 for all groups. Expression of β-galactosidase was normally distributed ($p > 0.05$) for all groups. Normal Q-Q plot assessment of this group determined it acceptable for analysis. There was homogeneity of variances, as assessed by Levene's test for equality of variances ($p = 0.0.057$).

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Zaniewska M, Filip M, Przegalinski E (2015) The Involvement of Norepinephrine in Behaviors Related to Psychostimulant Addiction. Current neuropharmacology 13:407-418.

Zhu L, Zhu J, Liu Y, Chen Y, Li Y, Huang L, Chen S, Li T, Dang Y, Chen T (2015) Methamphetamine induces alterations in the long non-coding RNAs expression profile in the nucleus accumbens of the mouse. BMC neuroscience 16:18.

Zurkovsky L, Sedaghat K, Ahmed MR, Gurevich VV, Gurevich EV (2017) Arrestin-2 and arrestin-3 differentially modulate locomotor responses and sensitization to amphetamine. Neuropharmacology 121:20-29.

Curriculum Vitea

REBECCA V. FALLON

born in the USA in 1986

AFFILIATION AND ADDRESS

Johns Hopkins University & National Institute on Drug Abuse

Email: becky.fallon@jhu.edu; fallon.nih@gmail.com

SUMMARY

A PhD Candidate with over ten years of experience researching neurobiology of motivated behavior and decision-making.

EDUCATION

1. **Johns Hopkins University** **Baltimore, MD**

   Partnership Program at the National Institutes of Health Ph.D. Oct 2017

2. **University of Rochester** **Rochester, NY**

   Major in Brain and Cognitive Sciences B.S. May 2008

   Minor in Economics

   Minor in Classical Civilization

3. **Anatolia School of Business** **Thessaloniki, Greece**

   Study Abroad in Finance Fall 2006

RESEARCH EXPERIENCE

1. Graduate Student, National Institute on Drug Abuse, mentored by Dr. Bruce Hope

   2012 – 2017

2. Research Specialist, Medical University of South Carolina, mentored by Dr. Gary Aston-Jones 2008 – 2011

3. Technician, University of Rochester Medical Center in the Neuroscience Behavioral Core, mentored by Dr. Troy Zarcone and Dr. Bernard Weiss 2007 – 2008

4. Student Intern, Smoker's Health Project of Rochester, NY Spring 2007

TEAMWORK AND LEADERSHIP EXPERIENCE

1. Global Scholar & Consultant for PreScouter, Inc. 2014-present
   a. Developed creative solutions to problems of three PreScouter clients by surveying latest scientific advances and integrating innovative technologies
2. Board member of Johns Hopkins Graduate Consulting Club 2016-2017
   a. Team leader and engagement recruiter for pro bono projects 2016 – present
   b. Director of Finance for JHGCC raising over $12k for events 2016-2017
3. Executive Board Member of Johns Hopkins Graduate Representative Organization
   a. Elected Chair of the GRO 2015-2016
   b. Elected Editor and Website Designer of the GRO Guide 2014 – 2015
   c. Elected Alumni Relations Chair 2013 – 2014
4. Teaching assistant for Johns Hopkins University undergraduate courses
   a. Human Origins, department of Psychological Brain Sciences Fall 2014
   b. Cellular Biology Lab, department of Biology Spring 2012
   c. Biochemistry Lab, department of Biology Fall 2011
5. Committee member for Johns Hopkins Biology Annual Retreat Oct 2014

6. Volunteer mentor for Baltimore City schools STEM development

a. STEM Achievement in Baltimore Elementary Schools 2014 – present

b. Hopkins Mentors to Inspire Diversity in Science 2011 – 2015

7. Commenced and ran a monthly Addiction Journal Club at NIDA 2013 – 2015

8. Director of events for JHU student-invited honorary lecture series 2012 – 2016

9. Initiated and organized a research internship for 12 undergraduates through Medical University of South Carolina 2010 – 2011

a. Mentored a College of Charleston undergraduate for one year on an award-winning biology honors thesis

DISTINCTION AND HONORS

1. Awarded $30,000 for *Graduate Student Incentive Grant* by NIDA 2012 – present

2. Awarded $1000 for 2015 *NIH Graduate Student Research Award* by NIH Jan 2015

3. Awarded a *Degree of High Distinction* for graduating top 10% in Brain and Cognitive Sciences department at University of Rochester May 2008

4. Awarded *Dean's Scholarship for Overall Excellence*, U. of Rochester 2004 – 2008

5. Awarded *UAA All-Academic Athlete* for swimming by UAA 2004 – 2007

MANAGEMENT EXPERIENCE

1. NIH Office of Intramural Education Course on Approaches to Mentoring Feb 2015

2. American Society for Cell Biology Mini-Course: Managing Science in the Biotech Industry at University of Pennsylvania Dec 2014

3. NIH Office of Intramural Education Course on Workplace Dynamics; focus on communication, leadership, self-awareness, conflict resolution, team skills, and diversity at NIH 2013 – 2014

PROFESSIONAL MEMBERSHIPS AND WORKSHOPS

1. Selected Participant: *Gordon Conference on Catecholamines* Aug 2015
2. Participant: *Achieving Gender Equality in Science Workshop* Fall 2014
3. Selected Participant: *Gordon Conference on Catecholamines* Aug 2013
4. Society member: *Society for Neuroscience* 2009—present
5. Board member: *Wiley-Blackwell Scientific Advisory Group* 2009—present
6. Selected participant: *Workshop in Cognitive Science and Cognitive Neuroscience*, at Institute for Research in Cognitive Sciences, U. of Pennsylvania June 2007

MANUSCRIPTS

1. S. Mahler, M. Hensley-Simon, P. Tashsili-Fahadan, R. LaLumiere, C. Thomas, **R. Fallon**, P. Kalivas, G. Aston-Jones. *Modafinil attenuates reinstatement of cocaine seeking: role for cystine-glutamate exchange and metabotropic glutamate receptors.* Addiction Biology. September 2012.

2. L.R. Whitaker, K.B. McPherson, P.E. Carneiro de Oliveira, **R.V. Fallon**, A. Bonci, B.T. Hope. *Associative learning drives the formation of silent synapses in neuronal ensembles of the nucleus accumbens*. Biological Psychiatry. August 2015.

3. **R.V. Fallon**, B.L. Warren, L.R. Whitaker, F.J. Rubio, B.T. Hope. *Characterization of Fos-expressing neuronal ensembles playing a causal role in amphetamine sensitization*. (in prep).

SELECT POSTERS AND PRESENTATIONS

1. A.M. Cason, **R.V. Fallon**, G. Aston-Jones. Effects of the orexin/hypocretin 1 receptor antagonist SB-334867 on sucrose-seeking in rats. Poster session presented at: SfN 2009. 39th Annual Conference of the Society for Neuroscience; 2009 Oct 17-21; Chicago, IL. Abstract No. 755-10.

2. **R.V. Fallon**, R.J. Smith, G. Aston-Jones. The effects of a history of chronic cocaine exposure and protracted abstinence on future cocaine self-administration in rats. Poster session presented at: SfN 2010. 40th Annual Conference of the Society for Neuroscience; 2010 Nov 13-17; San Diego, CA. Abstract No. 67-7.

3. **R.V. Fallon**, T. D. Smith, S.V. Mahler, G. Aston-Jones. Kappa-opioid receptor modulation of protracted withdrawal from cocaine: Nor-BNI effects on value of self-administered cocaine and on locomotor sensitization. Poster session presented at: SfN 2011. 41st Annual Conference of the Society for Neuroscience; 2011 Nov -16; Washington D.C. Abstract No. 942.11.

4. S. Drumm, **R.V. Fallon**, D. Moorman, G. Aston-Jones**.** Rats take more cocaine rewards than sucrose rewards in fixed-ratio self-administration choice task. Poster session presented at: Frontiers in Neuroscience Research Day. 12th Annual Conference of Neuroscience Institute; 2011 Apr 5; Charleston, SC. ***Award winner**

5. **R.V. Fallon,** F. Cruz, B.T. Hope. Assessing dendritic spine plasticity in transgenic rat nucleus accumbens neuronal ensembles activated during amphetamine sensitization. Poster session presented at: 44th Annual Conference of the Society for Neuroscience, 2014 Nov 14-19; Washington D.C. Abstract No. 811.13.

6. **R.V. Fallon,** J. Rubio, B.T. Hope. Assessing cellular alterations in Fos-expressing accumbens neurons after amphetamine sensitization. Poster session presented at: NIH Graduate Student Research Symposium. 10th Annual Conference of NIH; 2015 Jan 13th; Bethesda, MD. ***Award winner**

7. **R.V. Fallon**, J. Rubio, B.T. Hope. Defining Fos-expressing neurons and dendritic alterations in rat nucleus accumbens following amphetamine sensitization. Poster session presentation at: Gordon Research Conference: Fronteirs in Catecholamine Function from Synapses to Disease; 2015 Aug 9-15, Newry, ME.

8. **R.V. Fallon**, J. Rubio, B.T. Hope. Characterizing Fos-expressing neuronal ensembles in the nucleus accumbens after amphetamine sensitization in rats. Poster session presented at: Winter Conference on Brain Research. 49th Annual Conference; 2016 Jan 23-28, Breckenridge, CO.

9. **R.V. Fallon**, B. Warren, F. J. Rubio, B. T. Hope. Characterizing Fos-expressing neuronal ensembles in rat nucleus accumbens that are associated with amphetamine sensitization. Poster session presented at: 46th Annual Conference of the Society for Neuroscience, 2016 Nov 12-16; San Diego, CA. Abstract No. 837.04.